# Agilent ChemStation

# Understanding Your ChemStation



**Agilent Technologies**

# Notices

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## Research Use Only

**Not for use in Diagnostic Procedures.**

## Software Revision

This guide is valid for B.04.xx revisions of the Agilent ChemStation software, where xx refers to minor revisions of the software that do not affect the technical accuracy of this guide.

## Warranty

## Technology Licenses

## Restricted Rights Legend

## Safety Notices

### CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

### WARNING

**A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.**

# In This Guide...

This guide describes various concepts of the Agilent ChemStation. It is intended to increase your understanding of how the ChemStation works.

For information on using the ChemStation please refer to the general help system and the Online help "Tutorial".

## 1 Agilent ChemStation Features

This chapter describes the main components and features of the ChemStation.

## 2 Methods

This chapter describes the concepts of methods and how to work with them.

## 3 Data Acquisition

This chapter describes the concepts of Data Acquisition, data files, logbook, and more.

## 4 Integration

This chapter describes the concepts of integration the ChemStation integrator algorithms. It describes the integration algorithm, integration and manual integration.

## 5 Quantification

This chapter describes how ChemStation does quantification. It gives details on area% and height% calculations, external standard (ESTD) calculation, norm% calculation, internal standard (ISTD) calculation, and quantification of unidentified peaks.

## 6 Peak Identification

This chapter describes the concepts of peak identification.

# Contents

# 1
# Agilent ChemStation Features

**Agilent Technologies**

**1    Agilent ChemStation Features**
In This Guide...

This chapter describes the main components and features of the ChemStation.

# General Description

The ChemStations for GC, LC, LC/MSD, CE, CE/MSD, and A/D systems are instrument control, data acquisition and data evaluation systems for

- Agilent 7890A Gas Chromatographs,

- Agilent 6890N, 6890Plus and 6890A Gas Chromatographs,

- Agilent 6850 Gas Chromatographs,

- 5890 Series II Gas Chromatograph

- Agilent 1100/1200 Series modules and systems for LC,

- Agilent 1100 Series LC/MSD,

  Agilent 6100 Series Single Quad LC/MSD

- 1090 Series liquid chromatographs,

- 1046 FLD and 1049 ECD Detectors

- Agilent Capillary Electrophoresis system (CE)

- Agilent CE/MS system and

- Agilent 35900E dual channel analog-to-digital interface.

The software is designed to run on IBM-compatible personal computers under Microsoft® Windows XP Professional operating environment.

The software is sold as a single instrument basic ChemStation in five versions. All versions include data acquisition, instrument control, data analysis (integration, quantification and reporting), automation and customization for one analytical instrument. An instrument is defined as running on a single timebase, but can collect data from a number of different detectors simultaneously. The five versions are:

- a single instrument ChemStation for gas chromatography (GC) systems, product number G2070BA,

- a single instrument ChemStation for liquid chromatography (LC) systems, product number G2170BA,

- a single instrument ChemStation for capillary electrophoresis (CE) systems, product number G1601BA,

- a single instrument ChemStation for liquid chromatography/mass selective detector (LC/MSD) systems, product number G2710BA, and

- a single instrument analog-to-digital (A/D) ChemStation for analog data acquisition with external event control, product number G2072BA.

The instrument control capability of the ChemStation software may be expanded by purchasing additional instrument data acquisition and control modules to allow multiple instrument, mixed technique configurations.

## Additional Instrument Modules

The additional instrument modules are:

- additional GC instrument control and data acquisition module, product number G2071BA,
- additional LC instrument control and data acquisition module, product number G2171BA,
- additional CE instrument control and data acquisition module, product number G2172BA,
- LC/MSD instrument control, data acquisition, and data evaluation add-on module, product number G2715BA, and
- additional analog data acquisition module, product number G2073BA.

## Additional Data Evaluation Modules

The data processing capability of the ChemStations may also be expanded through the purchase of additional data processing modules for specialist applications:

- additional diodearray detector (DAD) spectral evaluation module, product number G2180BA,
- additional ChemStore sample organization and results database module, product number G2181BA, and
- LC/MSD deconvolution and bioanalysis data evaluation module, product number G2720BA, for use with the LC/MSD ChemStation only.

Up to four chromatography instruments may be configured on each ChemStation. If instruments with spectroscopy detectors (diode array detectors for liquid chromatography or capillary electrophoresis) are

configured, no more than two diode array detectors are supported on one ChemStation, and the number of supported instruments is restricted to three. When the ChemStation for LC/MS is used to control the Agilent 1100/1200 Series LC/MS module (optionally with one Agilent 1100/1200 Series LC or 1090 Series II LC), no other instruments are supported on the PC.

## Data Evaluation-only Products

There are also three data evaluation-only products available that may not have instruments configured. They are designed to be used for data evaluation in an office environment:

- the ChemStation for LC 3D data evaluation, product number G2190BA, includes diode-array spectral data evaluation and the capabilities of the ChemStation for data evaluation and

- The ChemStation for LC/MSD data evaluation, product number G2730BA, includes diode-array spectral data evaluation, mass spectral data evaluation, as well as the capabilities of the basic ChemStation for data evaluation.

# ChemStation Hardware

For details of ChemStation hardware, see *Installing Your ChemStation* manual.

# About the ChemStation Software

## Operating System

The ChemStation requires Microsoft Windows XP Professional SP3 or Windows Vista Business SP1 operating system.

The ChemStation Control Charts feature requires MicroSoft Excel.

## Methods and Sequences

The analytical method fully describes how a particular separation is performed. It contains all parameters for instrument control, data acquisition and evaluation, including integration, quantification and reporting. The system may be set up to acquire data from a number of samples by different methods. The control file for this sort of operation is called a sequence, and holds the individual sample information, references to the appropriate methods, and automatic recalibration specifications. For further information on methods and sequences, see "Automation" on page 26 and the online help system.

## System Configuration

The configuration of the instrument system is done through the configuration editor program. It allows you to define your instruments, their GPIB or LAN addresses, the directories for your data, sequences and methods and the color definition for the ChemStation software. For further information, see the handbooks supplied with the additional ChemStation modules.

# Data Model

The ChemStation software is designed around a data model based on a memory structure called a register. Registers are multipurpose structures that can hold analytical data and information for both two-dimensional information (for example, time/intensity) and three-dimensional information (for example, time/intensity/wavelength).

The ChemStation provides commands and functions to construct, expand, extract and, where it does not alter primary data, edit registers. For further information, see the *Macro Programming Guide* which is available as online help.

# File Naming Conventions

## Naming Conventions

The following rules enable the ChemStation to create and process valid names for files and directories:

The following characters are not allowed as part of a file or directory name:

- < > : " / \ | @ % * ? blank (spaces) etc.

If these characters are used in the installation folder, the reprocessing copy does not start, and if the character % is used in the installation folder, some 'Agilent Chemstation B.04.01' shortcuts do not work properly.

The following rules apply in addition:

**Table 1**    Restricted characters

| ChemStation parameter | Character |
|---|---|
| Method File Names: | % and . (decimal point) are not allowed |
| Data File Names (Prefix/Counter): | blanks are not allowed |
| Data Subdirectory and Sequence Subdirectory: | [] + = ; , . (decimal point); spaces are not allowed |

The following reserved device names cannot be used as the name of a file:

- CON, PRN, AUX, NUL

- COMx (where x is a number from 1 to 9)
- LPT1x (where x is a number from 1 to 9)

Also avoid these names followed by an extension (e.g. Nul.txt).

| **NOTE** | English, Japanese, and Chinese operating systems are used to test naming conventions. Agilent cannot give a support statement for non-English operating systems and their special characters. |
|---|---|

## Maximum Length of ChemStation file names and subdirectories

The Agilent ChemStation specifications for file names and subdirectories are listed below:

**Table 2**    Maximum Length of ChemStation file names and subdirectories

| DataFile/Subdirectory/Path | Max. Input Length | Auto append | Example |
|---|---|---|---|
| Data file name | 38 | . .D | Demodad.d |
| Data file name using prefix/counter | 15 | . .D | longname000001.d |
| Method Sequence Hypersequence Libraries Customized Report Templates | 40 | . M<br>. S<br>. HYP<br>. UVL<br>. FRP | def_lc.m<br>def_lc.s<br>def_lc.hyp<br>demodad.uvl<br>areapct.frp |
| Data file subdirectory | 40 | | demo (in sample info) |
| Data sequence subdirectory | 40 | | demo (in sequence parameters) |
| Sequence Data Container Name | 40 | | test_date_time (create using sequence preferences) |
| Data Path<br>Method Path<br>Sequence Path<br>Hypersequence Path<br>Libraries Path<br>Customized Report Template Path | 100 | 100 | c:\chem32\1\data<br>c:\chem32\1\methods<br>c:\chem32\1\sequence<br>c:\chem32\1\hyper<br>c:\chem32\speclib<br>c:\chem32\repstyle |

| NOTE | The toolbars displaying data file/sequence/method names have been resized to display up to 18 characters. |
|---|---|

All ChemStation logbooks report system messages in an extended format and information strings are printed over multiple lines. Certain reports, e.g. Sequence report, may truncate filenames to fit all information onto the report template.

## Software User Interface

The ChemStation user interface is designed into Views that group software functionality according to typical analytical tasks. The following three standard views are present in all software configurations:

- the Method and Run Control view for controlling and acquiring data from the instrument,
- the Data Analysis view for reviewing and reevaluating data that has been acquired,
- the Report Layout view for designing specific report layouts, and

Additional views are present if additional data evaluation modules have been ordered or for certain instrument configurations that support instrument diagnostics and verification procedures. A ChemStation Companion view is available for when it is desirable to have instrument operators run samples from an easy-to-use, preconfigured table.

The Navigation Pane contains the Navigation Button, to allow rapid switching between the ChemStation views, and the tree-based ChemStation Explorer. The contents of the ChemStation Explorer are view-dependent, giving access to different ChemStation elements.

Each view consists of a set of standard user elements including menus and toolbars. The standard toolbar provides rapid access to the common system specification information such as methods and sequences. The Method and Run Control view additionally incorporates a system status bar, a sample information area, that may be configured for single runs or automated runs, and a schematic instrument interface diagram for GC, CE and LC configurations. The schematic instrument interface diagram uses hot spots to

allow rapid access to instrument parameters and an animated graphical overview of the status of each analysis as it proceeds. The schematic instrument diagram may be turned off if it is not required, to save memory and other Windows resources.

The Data Analysis view extends the standard toolbar to specific data analysis modes including integration, calibration, reporting, annotation, signal comparison and additional specialized modes if the modules are installed. Each of these separate data analysis modes are supported with a mode-specific toolset.

The Report Designer view allows the user to graphically define the layout of a specific report style in a graphical object orientated fashion. It also uses a set of toolbars specific to this task.

## Data Acquisition

The status of the instrument is continually monitored and updated on the display, along with the elapsed run time of the analysis both when the software is a visible window and when it is iconized. The transactions that occur during the analysis, including any errors and the instrument conditions at the start and the end of the analysis, are recorded in the system's logbook, an extract of which is stored with every data file.

The instrument conditions, such as flow, temperature, pressure and solvent composition for liquid chromatographs may be recorded and stored with each data file. These instrument parameters can be displayed and plotted to testify to the quality of each analysis. The exact nature of the parameters recorded depends both on the technique and the capabilities of the configured instrument.

One or more display windows may be used to monitor the data being acquired by the instrument in real time. The data are displayed in real measurement units such as mAU, Volts, degrees or bar. The windows may each show multiple overlaid chromatographic/electropherographic signals or instrument parameters, such as pressure. The display default settings may be adjusted and are remembered by the system so users can set their own preferred settings as the instrument default. The window has zoom capability and the cursor may be used to display a specific signal's response at any point in time.

During an analysis, the complete functionality of the ChemStation can be used through the offline copy. While acquisition is running, the Data Analysis part of the online session of an instrument is not accessible, and data review has to be performed in the offline copy.

A snapshot function is available for users who wish to start processing data before the analysis is completed. The snapshot has to be taken in the offline copy of the instrument sessions and is immediately present for review.

The layout of the signal and status information windows, including the components of the schematic instrument interface diagram is saved automatically.

For further information on Data Acquisition, see "Data Acquisition" on page 59 and the online help system.

## Data Analysis — Display

The Data Analysis view extends the standard tool bar with task-grouped data analysis functions including integration, calibration, reporting, annotation, and signal comparison toolsets. The following key graphical operations are possible:

- single or multisignal displays selectable when loading the chromatogram/electropherogram,
- overlays of chromatogram/electropherogram from different samples,
- subtraction of one chromatogram/electropherogram from another,
- graphical vertical and horizontal alignment of signals to help visual comparison,
- signal inversion or mirroring to help visual comparison,
- graphical zoom and scrolling functions,
- adjustment of display attributes including selection of tick marks, baselines, axes, retention/migration times and compound names (the user can also select the font for the RT and compound labels, adjust the size and orientation of the display, select the display as overlaid or separated and select scaling factors),
- the chromatogram/electropherogram display may include graphical overlays of instrument parameters depending on the capability of the configured instrument,

- user-defined annotations may be interactively added to the display, with the selection of font, size, text rotation and color (once defined, the annotations may be graphically moved, edited or deleted),

- copy the display to the Windows clipboard in both metafile and bitmap format,

- a *pick mode* function to display the values of individual data points in detector units, and

- export of time/intensity digitized points to the Microsoft Windows clipboard.

## Data Analysis — Integration

The ChemStation integrator algorithm is the second revision of a new generation aimed at improved ruggedness, reliability and ease-of-use.

## Data Analysis — Quantification

The ChemStation's calibration mode of the data analysis view allows simultaneous display of:

- the signal or signals being calibrated with an indication of the current compound's retention/migration time window,

- the calibration table whose display may be configured from a comprehensive selection of calibration parameters, and

- the calibration curve for the compound being calibrated.

All the calibration mode windows are linked so that changes in one are automatically reflected in all the others. This mode allows graphical selection and modifications of the calibration data.

Quantification is based on %, Normalized %, External standard, External standard %, Internal standard and Internal standard % calculations calculated on either peak area or height. Calibrations may be multilevel and include multiple internal standard definitions. Calibration histories are automatically saved and can be used to weight the recalibration calculations.

For information on calibration and quantitation, see "Calibration" on page 141.

## Data Analysis — Data Review, Data Reprocess and Batch Review

The following two additional toolsets are available within the Data Analysis view :

- Navigation Table
- Batch Review

The Navigation Table makes possible several key graphical operations:

- standard table configuration features, such as sorting, drag-and-drop options, column selection, item grouping to specify a preferred navigation table configuration
- right mouse click functions to load a signal, overlay a signal, export data, print reports
- review signal details by expanding a line in the navigation table
- review signals and create ChemStation reports using either loaded methods or the individual data file method DA.M, for example, to review manual integration events
- reprocess sequence data (acquired sequence data from ChemStation Rev. B.02.01 and later)

The following key graphical operations are possible in the Batch Review:

- define automatic or manual review and reprocess of (calibrated) data files
- recalibration of calibration table
- review compound tables of calibrated methods
- create specific batch reports

## Data Analysis — Standard Reporting

A standard set of user-definable report styles for sample reporting can be selected from the report specification screen. Every standard report type contains standard information groups and optional information groups.

For more information on the report styles available, see "Using the ChemStation Reports" on page 213.

# Data Analysis — Specialized Reporting

Advanced reporting capabilities are also included in the ChemStation for applications that require a more specialized set of reports. These include statistics on separation quality, reports that include trend analysis between samples and user-defined report layouts.

## System Suitability Reports

System suitability reports enable users to report system performance parameters for individual analyses. There are three variations, or styles of these reports.

The standard template **Performance report** prints parameters for uncalibrated methods that include:

- retention/migration time,
- capacity factor (k'),
- peak area,
- peak height,
- symmetry,
- true peak width at half height,
- efficiency in plate numbers,
- resolution, and
- selectivity.

For calibrated methods the compound name and amount replace the peak area, height and selectivity columns.

The report header includes the standard header and footer, sample information block, the analytical column parameters and optionally a plot of the chromatogram/electropherogram.

The **Performance and Noise** style adds an evaluation of the signal noise, in up to seven user-defined evaluation ranges, to the data from the **Performance report** style. The noise parameters are reported as signal to noise ratios for each peak or calibrated compound and a noise table for each signal. Each noise table includes noise calculated by the six times standard deviation, peak to peak and ASTM methods as well as the wander and drift.

The **Extended Performance** style adds plots of each individual peak graphically showing the peak start and stop times, half width and baseline. This style includes the following parameters in addition to the ones reported by the standard Performance reports:

- area, height and amount,
- skew,
- excess,
- usp tailing factor,
- time interval between data points and number of data points over the peak,
- statistical moments (M0 to M4),
- peak width at half height calculated by the true, five sigma, tangent and tailing methods, and
- plate/column and plates/meter calculated by the peak width at half height, five sigma, tangent and statistical methods.

Users may define their own noise evaluation ranges and acceptable limits for these performance criteria. Values lying outside the user-defined acceptable limits are indicated on the report.

For more information on system suitability calculations, see "Evaluating System Suitability" on page 231.

## Sequence Summary Reports

Sequence summary reports are produced at the end of a series of automated analyses. Their range of application is from a brief summary of the samples analyzed to a detailed graphical repeatability or trend analysis of user-selectable parameters between different samples, analyzed by the same method.

For more information on sequence summary reporting, see the online help system and "Sequence Summary Reporting" on page 226.

## Customized Reports

A customized reporting design view is included in the ChemStation for users who want to define the exact content of their own reports. The user graphically defines a report layout which may include general sample information, signal, integration and quantitative analytical result information. The user may define individual elements, such as text, tables and graphics,

organize them in information sections and graphically adjust their relative position size and orientation of each defined element. The individual sections may be added, deleted, reordered and nested.

The user may define headers and footers to appear on every page, time stamps for the report and page numbering in the **page x of y** format. The information included in the report may be any ChemStation or user-defined parameter.

Once the report has been designed it may be associated with a particular method to make it the default report format for that particular type of analysis.

Customized reports may be output to the screen, printer and to a file. Reports to the screen include graphics.

For more information on the report layout, see the online help system.

### Control Chart Reports

A **Control Chart** feature is included with the ChemStation software. Once this feature is installed and selected, the user may automatically track a selected parameter of a compound each time a method is run. These parameters include: Amount, Response Factor, retention/migration Time, and Area.

For more information on these Custom and Control Chart reports, see the online help system.

## Utilities and Compatibilities

### General

The ChemStation can import and export data files in the andi (Analytical Data Interchange) chromatography format of the Analytical Instrument Association (AIA), revision 1.0, copyright 1992. Data import is supported at compliance level one (sample information and signal data) and data export at compliance level two (sample information, signal data and integration results).

The ChemStation includes commands and functions to support the dynamic data exchange (DDE) standard of the Microsoft Windows platform as both a DDE client and a DDE server. The command set includes commands to establish and terminate connections, transfer information in both directions and execute remote functions.

## Customization

The ChemStation can be customized using the powerful command set. These commands may be grouped to execute automatically a specific function; such a group is called a macro. Users writing macros may define their own variables, build in conditional or looping constructs, perform physical I/O including file handling and user interaction, nest their macros and schedule and exchange data with other MS-DOS or Microsoft Windows applications.

For more information on customization, see the *Macro Programming Guide* which is available as online help.

## Automation

The ChemStation can execute multimethod sequences.

The sequence parameter set may be defined to use automatically generated files or sequentially numbered files with a user-defined prefix of up to fifteen characters. The user may select to run full analyses or data reprocessing only sequences and can also select one of a series of technique specific shutdown commands or a user-defined shutdown macro that runs when the sequence terminates either by error or after all the analyses are completed.

The sequence table, or list of analyses to run, is built in a spreadsheet-like user interface that allows users to specify vial numbers and sample names, analysis methods, sample quantification parameters including sample amount, a multiplier and dilution factor, calibration specification, data exchange parameter LIMSID and the number of repeat injections. Depending of the configured instruments and modules, more fields will accessible, e.g. if an Agilent 1100/1200 LC system includes a fraction collector, the "Fract. Start" column will appear in the sequence table. The appearance of the sequence

table can be configured by the user. The user can jump between individual cells in the table and copy, cut or paste individual cells or entire rows or series of rows in order to build sequences efficiently and quickly.

Samples may be identified in the sequence table as unknowns, calibration or control sample types. The sample type determines any special data evaluation treatment of the sample:

- unknown samples are evaluated and reported according to the method specification,
- calibration samples are used to recalibrate the quantification component of the method as described below, and
- control samples are evaluated against the limits for each component defined in the method. If the results lie outside any specified parameter range the execution of the sequence will be halted.

Calibration samples may be defined as simple, cyclic or bracketed. Simple recalibrations mean a recalibration occurs each time a calibration sample is defined in the sequence. Cyclic recalibrations occur at defined intervals during analysis of a series of unknowns. In bracketing a series of unknown samples, two calibration sets are analyzed. The quantitative reports for the unknown samples are then calculated using a calibration table averaged between the two calibration sets.

The partial sequence functionality allows users to see the order of execution of the sequence and also select individual sample entries to rerun or re-evaluate. When re-evaluating data already acquired users can specify whether reprocessing uses the original sample quantification data or new data entered in the sequence's sample table.

Sequences may be paused to run single injection priority samples by another method, then restarted without disrupting the automation. Samples can be added to the sequence table while the sequence is executing.

Both the sequence and partial sequence tables may be printed.

For more information on sequences, see "Automation" on page 159, and the online help system.

# Good Laboratory Practice

The ChemStation is developed to internationally recognized design and development standards and has a number of features specifically to help users operating in a regulated environment. These features are in the area of complete method specification and verification that the methods are fit for their intended use, to check the operation of their system and ensure the traceability, originality and quality of the data.

## Development Process

The Certificate of Validation shipped with each software package documents the software development and testing steps executed as part of the development cycle. The development process is registered to the ISO 9001 quality standard. It is documented together with onsite revalidation protocols in the *Validation BinderAgilent ChemStation for LC*.

## Method Specification and Use

- Global methods — the complete instrument and data analysis specification is stored in one place. Methods include individual compound range specifications to check that quantification results are not applied outside the calibrated range.

- The method change history log allows users of a validated method to automatically record how and when a method was changed. Users may optionally add a comment reason to the change history log. The change history log is automatically stored as part of the method in a binary format. To prevent unauthorized access to the records, it is protected by the user access scheme, described below. The change history log may be viewed and printed.

- Limits may be assigned on a compound-by-compound basis in each method for a number of chromatographic/electropherographic and system performance parameters, as described in the data analysis quantification section. Results exceeding these parameter ranges are used to control the execution of automated sequences as described in the automation section. They are indicated on the appropriate analysis report.

- System performance or suitability reports (see the Reporting section above) provide detailed analysis of the separation quality.

The ChemStation may be configured for restricted access for two user access levels, an operator and manager level. The manager level may be password protected and allows access to the complete ChemStation functionality. The operator level restricts the user to key functionality and executing defined analytical methods. The operator level is intended for use in routine laboratories and specifically prevents users from modifying and creating new methods.

## Method Robustness

Sequence summary reports (see "Sequence Summary Reporting" on page 226) provide a means to test methods for robustness. The extended format reports for user selected criteria, are reported as trend charts and may be used to determine the realistic operation limits. These limits can then be incorporated in the method to ensure, through the analysis of control samples, that the method is operating within specifications.

## System Operation

The ChemStation verification kit, which is part of the standard software, automatically checks for the correct installation and operation of the data evaluation parts of the software by comparing results generated when the test is executed against prerecorded known values. The verification kit allows users to define their own data files and methods to be the basis of the test.

## Data Traceability, Originality and Quality

Run-time logbook provides a transaction log of the complete system. It also records any unusual events (such as errors or parameter changes made during a run) as well as the instrument conditions before and after each analysis. A copy of the relevant logbook extract is saved with each data file.

The actual instrument conditions, such as pressure, flow, and temperature, that occurred during each analysis are also recorded if the configured instrument supports this capability. This data can be subsequently displayed graphically with the chromatogram/electropherogram to show the actual instrument conditions during that particular analysis, as well as being included on the report.

Methods saved with the data file record the actual method at the time of the analysis and allows the complete reconstruction of the reported data at a later date. The method is saved at the completion of all the analytical steps.

All reports have time stamps and traceable page numbering (*page x of y* pagination style). The user may select the level of detail in each report ranging from simple summary reports to complete system details (see the Reporting section above).

GLP Save register files, specified as part of the method configuration, save all the original data, including sample information, data analysis method, chromatographic/electropherographic signals, instrument conditions, integration and quantification results, report data and the run logbook in one checksum protected binary file. This is an uneditable binary format that ensures the originality of the results.The file includes a revisioning scheme that indicates if data has been reprocessed.

Control sample types may be defined in the sequence table and used to automatically check the instrument performance against quality control sample results when the instrument is running unattended. Results that are outside the user-specified acceptable range will stop the automatic execution of the instrument.

# Instrument Control

The instrument control capability of the ChemStation may be expanded through the purchase of additional instrument modules to allow multiple instrument, mixed technique configurations. For further information, see the handbook(s) supplied with the additional ChemStation modules.

## Networking

The ChemStation is tested and supported with Agilent's LanManager software, Microsoft Windows XP Professional and Microsoft Windows Vista Business products based on the IEEE 802.3 CSMA/CD specification. It should be compatible with any networking software compatible with the programming standards of Microsoft Windows.

These products enable the ChemStation to share physical devices such as plotters and printers with other laboratory computers as well as sharing information such as data files and methods.

### Client/Server

The ChemStation software may be installed on a suitable network server and downloaded onto the client PCs as required. Each client specific configuration ensures a suitable environment for different techniques and individual users while the centralized software installation relieves the burden of managing many copies of the same ChemStation installation in one work environment.

### LAN-based instrument control

The ChemStation software provides LAN-based instrument control and data acquisition for the Agilent 7890 GC, the Agilent 6890 GC, the Agilent 35900E A/D control module and the Agilent 1100/1200 Series LC. You can easily control and monitor instruments by connecting them to a LAN on which the ChemStation PC resides. This arrangement allows the ChemStation PC to be located remotely from the instruments it controls.

# Documentation

The documentation set contains specific sections on:

- Installing and learning the ChemStation software,
- Using the ChemStation software,
- Understanding the principles of how the software works, and
- Customizing the ChemStation.

## Installing and Learning

Each ChemStation software product comes with an installation manual that includes details of the key steps in PC hardware and software requirements, instrument interface installation, ChemStation installation and installation qualification. The installation manual is specific to the purchased configuration and may include troubleshooting, system records and system maintenance advice.

## Using the Software

Two additional categories of online information are designed for the routine user.

The ChemStation includes comprehensive, Windows-style, context-sensitive and indexed online help. This system gives detailed explanations of every screen and the meaning of the parameters on that screen. The detailed explanations are backed up by graphics where appropriate, and may be copied to the Windows clipboard for incorporation into the users' own documentation, or for printing.

An Online Tutorial included in the online help enables you to learn the software while working on your own methods and data. It provides an overview of the first steps in data acquisition and data analysis.

The How To part of the online help also includes check lists of the more complex technique-specific and common chromatography tasks to help less frequent users who want to be sure they set up the system correctly. These checklists are linked directly to the detailed online help information.

## Understanding the Principles

The *Understanding Your ChemStation* manual documents the principles of the software operation and the algorithms used in the data manipulations.

## Customization

Sophisticated users who wish to customize the operation of the ChemStation, or who want to build in additional features, may do so by writing macros.

The primary reference manual, *Macro Programming Guide* which is available as online help, has a comprehensive set of functional examples backed up by a complete description of the internal data types and structures.

The commands help file, accessed directly from the ChemStation's Help menu or the Show Command dialog box, is the programmer's function reference. It includes syntax and parameter explanations with example macros illustrating the use of many of the commands. By virtue of being online, the users can copy the examples and command syntax directly into their own macro source files.

# The ChemStation Directory Structure

The following example shows the directory structure of the ChemStation. It comprises generic directories that are shared by all configured instruments and instrument-specific directories. The software installation program creates a subdirectory of the ChemStation directory (by default CHEM32) for each configured instrument with the instrument number. Inside this subdirectory, the data, methods and sequences for this instrument are stored by default. Additional subdirectories for data, methods and sequences can be added using Preferences. Within the ChemStation Explorer, is it then possible to navigate to the newly added locations in order to load data, methods and sequences. The new locations are also available in each selection box within the ChemStation menu items (for example, for the path settings in the Sequence Parameters).

The ChemStation subdirectories are as follows:

```
CHEM32 ──────┬────── REPSTYLE
             ├────── CORE ──────┬────── PICTURES
             │                  ├────── Language
             │                  ├────── helpenu
             │                  ├────── 1024
             │                  ├────── 800
             │                  └────── ToolbarSupportFiles
             │
             ├────── LC
             ├────── GC
             ├────── CE
             ├────── MS
             ├────── IQT
             ├────── DRIVERS ──────┬────── Language
             │                     ├────── 800
             │                     ├────── ToolbarSupportFiles
             │                     ├────── GCI
             │                     └────── Rapid
             │
             │                  ┌────── TEMP
             ├────── 1 ─────────┼────── DATA ────── DEMO
             │                  ├────── METHODS ────── DEMO
             │                  ├────── SEQUENCE
             │                  └────── VERIFY
             │
             └────── speclibs
```
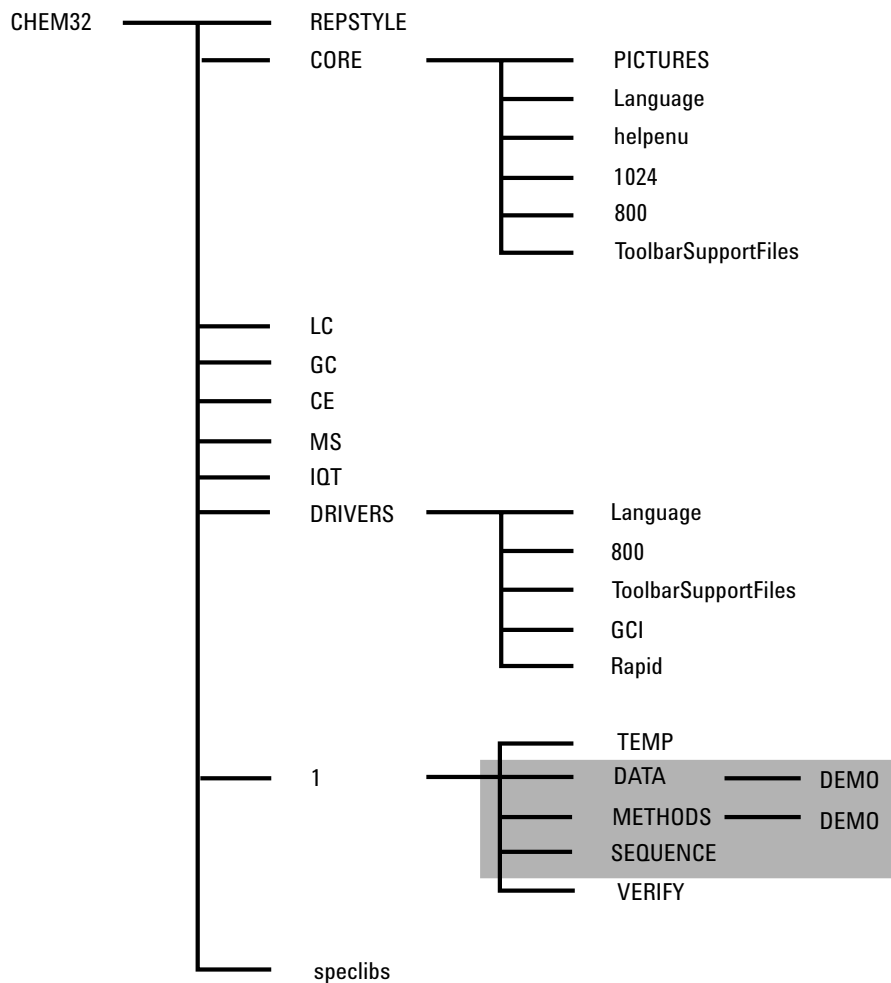
**Figure 1**    ChemStation Directory Structure

**Table 3** ChemStation subdirectories

| Directory | Contents |
|---|---|
| Chem32 | The directory comprises the programs to configure and start the ChemStation software. It must be part of the PATH variable. This directory is added automatically by the installation program unless you provide an alternative. |
| REPSTYLE | Used for report templates defined using the Report Template Editor. |
| CORE | Used for the core components of the software shared by all chromatographic/electropherographic instrument configurations. This is the working directory of the ChemStation. |
| PICTURES | Comprises graphics needed by the ChemStation. |
| Language | Used for the language-specific code of this part of the software. |
| 1024, 800, ToolbarSupport Files | Comprise initialization files for the graphical user interface. Do not change. |
| helpenu | Used for the US-English version of the help files for the appropriate software part. |
| BACKUP | Used for backup copies of old files during installation. |
| DRIVERS | Comprises instrument drivers. |
| 1 | Used for the configured instrument (1 to 4). This subdirectory comprises five additional subdirectories: DATA, METHODS, SEQUENCE, VERIFY, and TEMP. |
| DATA | Comprises the default result directories of your analyses. It can also comprise more subdirectories if you structure the disk with subdirectories as you work, by defining them using the Sample Information or Sequence Parameters dialog box. Result directories are identified by a name with a .D extension. For more information on the structure of data files, see "Data Acquisition" on page 59. Additional data paths can be added using Preferences. |
| METHODS | Comprises the default master method directories that have a .M extension. For further details on the contents, see "Method Directory Structure" on page 51. Additional method paths can be added using Preferences. |

**Table 3**     ChemStation subdirectories

| Directory | Contents |
| --- | --- |
| SEQUENCE | Comprises the default path for sequence templates. The sequence templates in these directories have a .S extension. Additional sequence paths can be added using Preferences, see "Preferences - Sequence Tab" on page 163 and "Sequence Parameters" on page 165. |
| VERIFY | Comprises data files, methods, and the results of data processing stored in register (.REG) files. These files execute the ChemStation verification procedure described in the online help. One set of data, method, and register files is used for each verification test. |
| TEMP | The TEMP subdirectory comprises temporary work files and the logbook files. For example, for Instrument 1 the online logbook is called INSTR1.LOG and the offline logbook is called INSTR1-2.LOG. |
| LC, GC, CE, MS | Instrument driver-specific code such as INI files. |
| IQT | Comprises files the files needed for IQT report creation. |
| speclibs | Comprises spectral libraries. |

# Navigation Pane

A Navigation Pane, available on the left side of all ChemStation views, is designed to speed access to many key ChemStation elements, as well as enabling quick switching between views. The Navigation Pane contains the tree-based ChemStation Explorer and a configurable button area. It also includes an autohide feature so that the ChemStation workspace is not compromised, and offers standard features such as resizing, and re-arranging of the navigation button area.

## Navigation Buttons

The Navigation Buttons allow the ChemStation view to be switched by clicking on the specific Navigation Button. The Navigation Button section can be minimized, expanded and re-arranged.

## ChemStation Explorer

The contents of the Navigation Pane are view-dependent. For Method and Run Control, Data Analysis and Report Layout, the ChemStation Explorer allows you to navigate to the different ChemStation elements. By default, these elements for data, methods and sequences are based on the configuration editor settings. The locations for these items can be expanded, and new nodes for methods, sequences, data location can be specified using the "Preferences" option in the view menu.

**Table 4**    Navigation Pane Items

| Navigation Buttons | ChemStation Explorer Elements |
| --- | --- |
| Method and Run Control | Sequence templates / Master methods |
| Data Analysis | Data / Master methods |
| Report Layout | Master methods |

**Table 4**    Navigation Pane Items

| Navigation Buttons | ChemStation Explorer Elements |
| --- | --- |
| Verification (LC and LC/MS) | Verification view-specific shortcuts |
| Diagnosis (LC and LC/MS) | Diagnosis view-specific shortcuts |
| Tune (LC/MS) | Tune view-specific shortcuts |

# 2
# Methods

This chapter describes the concepts of methods and how to work with them.

**Agilent Technologies**

# What is a Method?

A method comprises all the parameters for acquisition and data analysis together with pre- and post-run tasks for a given sample, if they are needed.

The available methods (*.m) files are visible in the ChemStation Explorer. For quick and easy navigation, you can add additional method locations to the ChemStation Explorer selection tree using the **Paths** tab of the **Preferences** dialog box.

# Parts of a Method

A method is identified by a name of up to forty alphanumeric characters. The file name always has the .M extension to identify it as a method. Methods are stored as directories that contain individual files relating to the components of the method.

Each method comprises four components:

- method information,
- instrument control,
- data analysis, and
- run-time checklist.

## Method Information

This section is used to define descriptive information about the method.

## Instrument Control

Defines parameters that control the instrument or its components. With an LC instrument, parameters, such as mobile phase composition, flow rate, injection volume, detector wavelength, and so on, control the pump, the injector and the detector. With a GC instrument, parameters such as inlet temperature, inlet pressure, packed column flow setting, and so on control the instrument.

## Data Analysis

Defines parameters that control the data processing.

### Signal Details

Defines signals and their properties used for data evaluation.

### Integration Events

Defines timed events that will occur at specific retention/migration times on a chromatogram/electropherogram. These timed events can be used to change the way the signal is integrated.

### Peak Identification

Defines data processing parameters associated with the identification of peaks in the chromatogram/electropherogram.

### Peak Quantification

Defines data processing parameters that affect the quantification calculations which determine the amount or concentration of sample component corresponding to each peak.

### Calibration and Recalibration

Defines data processing parameters that affect the calibration and how often calibration is done.

### Custom Fields

Defines the properties of sample or compound related custom fields that are available for the method. The custom fields allow to add custom information to a sample or a compound in a sample.

### Report

Defines the format of the report that is printed after a run.

# Run-Time Checklist

Defines which parts of the method are executed when the method is run.

You can use the run-time checklist to:

- acquire, store and process data to produce a report,
- execute only a portion of the method,
- acquire and store data without analyzing it,
- reanalyze existing data files,
- use your own macros for data analysis, pre- and post-run processing, and
- save the analysis results in a register for GLP purposes.

# Status of Methods

A method can exist in two states: as a stored method, or as the current loaded method.

## Stored Methods

These are methods stored on the computer disk. Stored methods have a name with up to forty alphanumeric characters followed by the extension .M. Methods are stored in the ChemStation in up to three locations:

- The Master Method is stored in a methods subdirectory, available in a Methods node of the ChemStation Explorer, and is not directly associated with any data container.

- When a sequence is run (using the option **Unique Folder Creation ON** (see "Preferences - Sequence Tab" on page 163), copies of all the master methods used in the sequence are stored in the sequence data container along with the sequence data files. These methods are directly linked to the sequence, and are used as well when the sequence is reprocessed. Changes to these methods are not propagated to the master methods. Changes are propagated to the Sequence Container Method as well to the individual methods (DA.M) during the sequence is reprocessed. If you now want to use the updated sequence container method for data acquisition, you need either to

  - copy this method from the sequence data container to one of the defined method paths or

  - use the **save as** option to save the updated method as a master method.

    The new/updated method is then available in the ChemStation Explorer in the method view as a master method.

- In addition, two copies of the method used to run a sample are stored with the data files - the data file individual methods ACQ.M and DA.M. ACQ.M is the acquisition method, DA.M is the data analysis method. DA.M is the method that is loaded along with the data file if the **Load DA method from data file** checkbox in the Signal Options tab of the Preferences dialog box is

selected. Changes to this method (for example, timed integrations events) are specific to the associated data file, and are not propagated to the sequence method or the master method.

The navigation table offers an update possibility for individual and sequence methods to their corresponding sequence or master method by right-mouse click on the method line item. Executing the Update Master Method / Update Sequence the data analysis parameters of the chosen method are copied over to the corresponding Master/Sequence method. The method history of the target method will reflect this automatic update.

## Current Method

When a stored method is recalled from the disk it becomes the current method. There is always a current method in memory. When the ChemStation is started for the first time, the default method supplied by Agilent Technologies is always loaded as part of the startup process. For example, this may be one either:

• DEF_LC.M for an LC instrument

• DEF_GC.M for a GC instrument

• DEF_CE.M for a CE instrument

A copy of the default method is placed in memory and becomes the current method. You can load a different method at this point and it will become the current method.

# Creating Methods

Creating a new method always means modifying the current method and saving the modifications under a new method name. Be aware that when the current method is changed, the disk version remains unchanged until you save your changes.

You have a choice of how to create a method. You can create a method to do either one or all parts of an analysis. For example, you can create a method to do only data acquisition. When you are ready to analyze the data and generate a library search report, you can then modify the method to do these data processing tasks.

**NOTE**    When you load a method to run a sample, always load a master method from a method node in the ChemStation Explorer. You should avoid using sequence methods (from a sequence data container) or individual methods associated with data files (ACQ.M or DA.M).

**NOTE**    Do not delete the default method (DEF_LC.M, DEF_CE.M or DEF_GC.M). These method files are used as templates to create new methods.

# Editing Methods

You can edit an existing method using the Edit Entire Method item of the Method menu. You are guided through all the method dialog boxes and at the end you can save the method. This process is shown below:

Method on disk

Load Method

Method loaded into memory, becomes current method

Select 'Edit Entire Method' from the Method menu

Edit Method

Save Method [As]

Save to new name               Save to same name

Creates new method             Overwrites current method
on disk
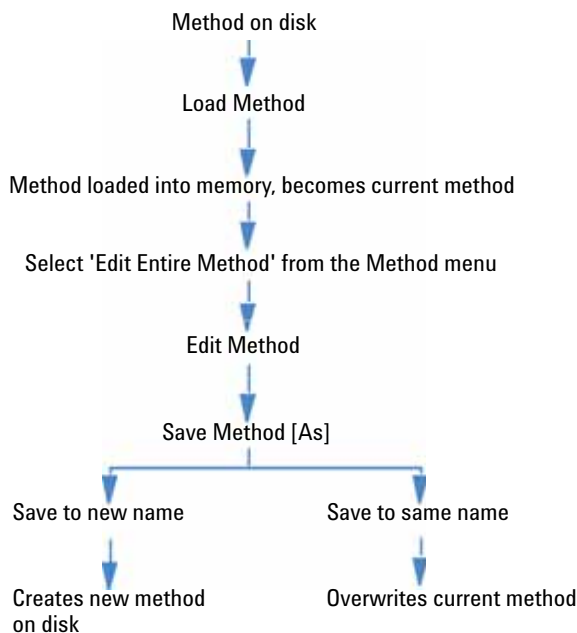
**Figure 2**     Editing Method

## Method Parts to Edit

Each method comprises four components that can be edited separately.

Some of the following subsections refer to specific dialog boxes, and some are generalized descriptions.

- *Method Information* comprises:
  - a text description about the method.

- *Instrument Control* depends on the configuration, and can comprise, for example:
  - oven parameters,
  - injector parameters, and
  - detector parameters.
- *Data Analysis* comprises:
  - signal details,
  - integration parameters,
  - quantification parameters,
  - calibration parameters,
  - custom field setup parameters, and
  - reporting parameters.
- *Run Time Checklist* comprises:
  - the parts of the method that will be executed.

| NOTE | Remember that methods can be stored in three locations in the ChemStation. Be sure that you are editing the correct method. |
|------|---|

# Method Directory Structure

A method comprises a group of files stored in the method directory.

The methods subdirectory comprises all the method subdirectories that have a .M extension. Additional methods subdirectories can be added using the preference settings.

Method files with the .MTH extension contain parameter sets and are in UNICODE format. The file INFO.MTH comprises the method control parameters.

Method files containing the instrument parameters have the name of the related analytical module. For example:

**Table 5**    Method file examples

| | |
|---|---|
| LC1090.MTH | Comprises the 1090 acquisition method. |
| GC5890.MTH | Comprises the 5890 acquisition method. |
| HPCE1.MTH | Comprises the acquisition method for the Capillary Electrophoresis. |
| DAD1.MTH | Comprises the acquisition method for the HP 1090. |
| FLD1.MTH | Comprises the acquisition method for the HP1046 fluorescence detector |
| ECD1.MTH | Comprises the acquisition method for the HP1049 electrochemical detector |
| ADC1.MTH | Comprises the Agilent 35900 acquisition method. If two identical instruments are configured, the method files are called ADC1.MTH, ADC2.MTH. |
| DAMETHOD.REG | For data evaluation. |
| LALS1.REG | Comprises parameters for the Agilent 1100/1200 Series autosampler. The method files for the other Agilent 1100/1200 Series modules follow the same convention lxxx1.reg where xxx is the module acronym. |

# What Happens When a Method is Run?

The run-time checklist dialog box specifies the parts of the method to execute when a run is started.

There are eight parts to the run-time checklist:

- pre-run command or macro,
- data acquisition,
- standard data analysis,
- analysis method for second signal (GC only),
- customized data analysis,
- save GLP data,
- post-run command or macro, and
- save copy of method with data (RUN.M).

When running a method the specified parts of the method defined in the Run Time Checklist dialog box are executed.

## Method Operation

The figure shows an overview of the ChemStation status during the method operation, where *all* parts of the Run Time Checklist are selected.

| NOTE | Please note, that in case of the mode "Unique Folder Creation OFF" no ACQ.M and DA.M are generated. For more details please refer to "Preferences - Sequence Tab" on page 163. |
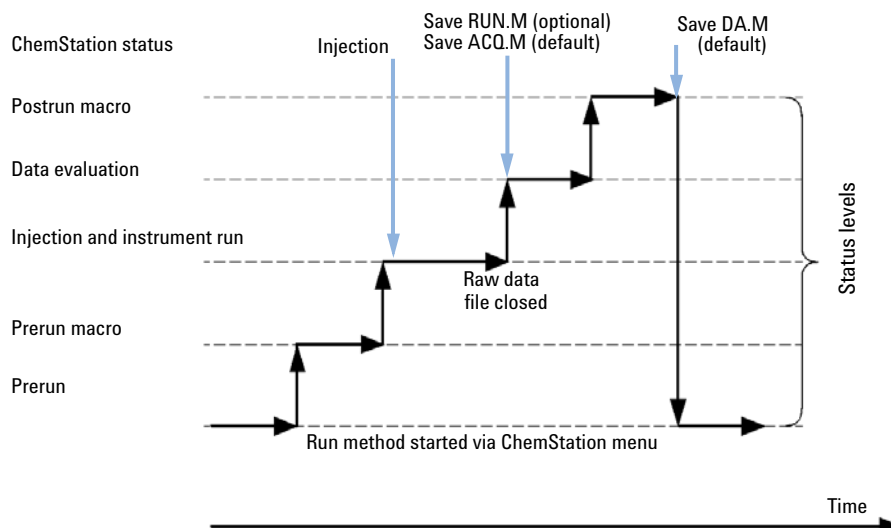
**Figure 3** Method Operation

# Pre-run Command or Macro (Run Time Checklist)

If a pre-run command or macro is specified, it is executed before the analysis is started. This part is typically used for system customizing in conjunction with other software packages.

# Data Acquisition (Run Time Checklist)

- All parameters are set to the initial conditions specified in the current method.
- If specified the injection program is executed and an injection is made from the currently defined vial.
- The monitor display shows the progress of the analysis including chromatographic/electropherographic information, and spectral data if available.
- Data are acquired and stored in a data file.
- When the Data Acquisition is finished, copy of the currently executed method is stored as ACQ.M for the data file by default.

# Data Analysis (Run Time Checklist)

When the stop-time has elapsed, the analysis is finished and all rawdata is stored on the computer's hard disk. The data analysis part of the software starts when all the rawdata is stored.

## Integration

- chromatogram/electropherogram objects in the signal are integrated as specified in the Integration Events dialog box.
- The start of the peak, the peak apex, retention/migration time and the end of the peak are determined.
- Baselines are defined under each peak to determine final peak height and area.
- The integration results are created as an Integration Results list.

## Peak Identification and Quantification

- Using retention/migration times and optional peak qualifiers, the software identifies the peaks by cross-referencing them with known components defined in the calibration table.
- By using peak heights or peak areas the software calculates the amount of each detected component using the calibration parameters specified in the Calibration Table.

## Spectra Library Search (ChemStations for LC 3D, CE, CE/MS and LC/MS Systems Only)

For all peaks that have UV-Visible spectra available, an automated search of a predefined spectral library may be done to identify the components in the sample based on the UV-Visible spectra. See *Understanding Your Spectral Module* for details.

### Peak Purity Checking (ChemStations for LC 3D, CE, CE/MS and LC/MS Systems Only)

For a peak with UV-Visible spectra, you can calculate a purity factor for that peak and store it in a register. Peak purity may be determined automatically at the end of each analysis as part of the method, if the Check Purity box is checked when specifying an automated library search or when selecting an appropriate report style. See *Understanding Your Spectral Module* for details.

### Print Report

A report is generated with identities and amounts of components detected in the run.

## Customized Data Analysis (Run Time Checklist)

Enables you to run your own customized macros to evaluate your analytical data.

## Save GLP Data (Run Time Checklist)

Saves the binary register GLPSave.Reg together with the data analysis method in the default data file subdirectory. This feature is designed to help prove the originality of the data and the quality of the individual analysis.

The GLPSave.Reg binary file contains the following information in a non-editable, checksum-protected, register file:

- key instrument set points (can be graphically reviewed),
- chromatographic or electropherographic signals,
- integration results,
- quantification results,
- data analysis method, and
- logbook.

These data are saved only if the Save GLP Data feature is activated by checking the check box in the runtime checklist. You can review, but not edit, GLP data in the data analysis menu of the ChemStation.

## Postrun Command or Macro (Run Time Checklist)

If a postrun command or macro is specified it is executed after the data evaluation, for example, copying data to a disk for data backup.

## Save Copy of Method with Data (Run Time Checklist)

This is done after data acquisition, and only if Data Acquisition is activated in the Run Time Checklist. It copies the *current* method to the data directory, named as RUN.M

## Save Copy of Method as DA.M with Data (ChemStation Default)

Depending on the marked items in the **Run Time Checklist**, a copy of the *current* executed method is saved as DA.M along with the data file. This is done the execution of the last marked item in the **Run Time Checklist**, in general after the **Standard Data Analysis** part.
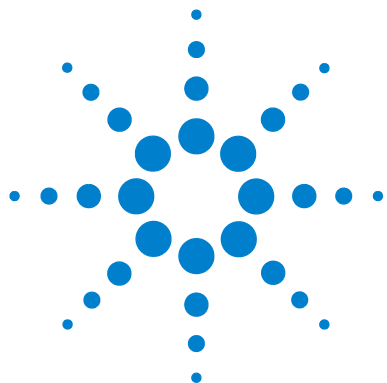
# Method Operation Summary

The following list shows the flow of the method operation when all parts of the Run Time Checklist are selected.

1 *Prerun Command Macro*

Does a task before the analysis is started.

2 *Data Acquisition*

Does injector program.

Injects sample.

Acquires raw data.

Stores data.

3 *Save Copy of Method with Data* (RUN.M) - optional by Run Time Checklist

4 *Save Copy of Method with Data* (ACQ.M) - ChemStation default

5 *Data Analysis (Process Data)*

Loads data file.

Integrates data file.

Identifies and quantifies peak.

Searches spectral library if available.

Checks peak purity if available.

Prints report.

6 *Customized Data Analysis*

Executes your macros.

7 *Save GLP Data*

Saves binary register GLPSave.Reg

8 *Postrun Command Macro*

Does a task after completion of the analysis, for example, generates a customized report.

9 *Save Copy of Method with Data* (DA.M) - ChemStation default

# 3
# Data Acquisition

This chapter describes the concepts of Data Acquisition, data files, logbook, and more.

**Agilent Technologies**

# What is Data Acquisition?

During data acquisition, all signals acquired by the analytical instrument are converted from analog signals to digital signals in the detector. The digital signal is transmitted to the ChemStation electronically and stored in the signal data file.

The available data (*.d) files are visible in the ChemStation Explorer. For quick and easy navigation, you can add additional data file locations to the ChemStation Explorer selection tree using the Paths tab of the Preferences dialog box.

# Data Files

A data file comprises a group of files, by default stored in the DATA directory as a subdirectory with a data file name and a .D extension. A data file name is can be defined manually using 40 characters including the extension. Each file in the directory follows a naming convention. Additional data directories can be added using the Preferences settings.

**Table 6**     Data files

| Name | Description |
| --- | --- |
| *.CH | Chromatographic/electropherographic signal data files. The file name comprises the module or detector type, module number and signal or channel identification. For example, ADC1A.CH, where ADC is the module type, 1 is the module number and A is the signal identifier and .CH is the chromatographic extension. |
| *.UV | UV spectral data files. The file name comprises the detector type and device number (only with diode array and fluorescence detector). |
| REPORT.TXT, REPORT.PDF | Report data files for the equivalent signal data files. |
| SAMPLE.MAC | Sample information macro. |
| SAMPLE.MAC.BAC | Backup of the original sample information macro. This file is created when the original sample parameters (like multipliers) are updated during reprocessing. |
| RUN.LOG | Logbook entries which have been generated during a run. The logbook keeps a record of the analysis. All error messages and important status changes of the ChemStation are entered in the logbook. |
| LCDIAG.REG | For LC only. Contains instrument curves (gradients, temperature, pressures, etc.), injection volume and the solvent descriptions. |
| ACQRES.REG | Contains column information. For GC it also contains the injection volume. |
| GLPSAVE.REG | Part of the data file when Save GLP Data is specified. |
| M_INTEV.REG | Contains manual integration events. |

The method can be stored with the result files (run.m). In such cases the method directory is stored as a subdirectory in the data file directory, using the option "Save method with Data" in the Run-Time-Checklist.

For data acquired with ChemStation B.02.01 revision and higher, each data folder (*.D) contains the following two methods folders

- acquisition method (ACQ.M) for each individual data file
- data analysis method (DA.M) for each individual data file

The individual acquisition method ACQ.M is intended to preserve the acquisition parameters, so it is recommended that you do not change this method during future data review activities. DA.M is the individual data analysis method for the specific data file. In the case of, for example, calibration table updates, the DA.M methods differ for each of the runs. With ChemStation B.03.01 or higher, this behavior can be switched off by disabling Unique Folder Creation.

# Online Monitors

There are two types of online monitors, the online signal monitor and the online spectra monitor.

## Online Signal Monitor

The online signal monitor allows you to monitor several signals and, if supported by the associated instrument, instrument performance plots in the same window. You can conveniently select the signals you want to view and adjust the time and absorbance axis. For detectors that support this function a balance button is available.

You can display the absolute signal response in the message line by moving the cross hair cursor in the display.

## Online Spectra Monitor

The online spectra monitor is only available for ChemStations that support spectra evaluation. It shows absorbance as a function of the wavelength. You can adjust both the displayed wavelength range and the absorbance scale.

# Logbook

The logbook displays messages that are generated by the analytical system. These messages can be error messages, system messages or event messages from a module. The logbook records these events irrespective of whether they are displayed or not. To get more information on an event in the logbook double-click on the appropriate line to display a descriptive help text.

# Status Information

## ChemStation Status

The ChemStation Status window shows a summary status of the ChemStation software.

When a single analysis is running:

- the first line of the ChemStation Status window displays run in progress,
- the second line in the status window displays the current method status, and
- the raw data file name is shown in the third line together with the actual run time in minutes (for a GC instrument, files for front and back injector are also displayed).

The Instrument Status windows provide status information about the instrument modules and detectors. They show the status of the individual components and the current conditions where appropriate, for example, pressure, gradient and flow data.

## Status Bar

The graphical user interface of the ChemStation system comprises toolbars and a status bar in the Method and Run Control View of the ChemStation. The status bar comprises a system status field and information on the currently loaded method and sequence. If they were modified after loading they are marked with a yellow cogwheel. For a Agilent 1100/1200 Series module for LC a yellow EMF symbol reminds the user that usage limits that have been set for consumables (for example, the lamp) have been exceeded.

# System Diagram

If supported by the configured analytical instruments (for example, for the Agilent 1100/1200 Series modules for LC or the Agilent 6890 Series GC) you can display a graphical system diagram for your ChemStation system. This allows you to quickly check the system status at a glance. Select the System Diagram item from the View menu of the Method and Run Control View to activate the diagram. It is a graphical representation of your ChemStation system. Each component is represented by an icon. Using the color coding described below the current status is displayed.

**Table 7**    System diagram color coding

| Color | Status |
|-------|--------|
| gray | inactive or off |
| yellow | not ready |
| green | ready |
| blue | run |
| red | error |

In addition, you can display listings of actual parameter settings. Apart from a status overview, the diagram allows quick access to dialog boxes for setting parameters for each system component.

See the instrument part of the online help system for more information on the system diagram.

# 4

# Integration

**Agilent Technologies**
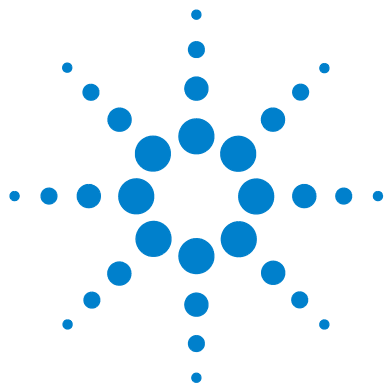
This chapter describes the concepts of integration the ChemStation integrator algorithms. It describes the integration algorithm, integration and manual integration.

# What is Integration?

Integration locates the peaks in a signal and calculates their size.

Integration is a necessary step for:

- quantification,
- peak purity calculations (ChemStations for LC 3D, CE, CE/MS and LC/MS Systems only), and
- spectral library search (ChemStations for LC 3D, CE, CE/MS and LC/MS Systems only).

# What Does Integration Do?

When a signal is integrated, the software:

- identifies a start and an end time for each peak and marks these points with vertical tick marks,
- finds the apex of each peak; that is, the retention/migration time,
- constructs a baseline, and
- calculates the area, height and peak width for each peak.

This process is controlled by parameters called integration events.

# The ChemStation Integrator Algorithms

The ChemStation integrator algorithm is the second revision of a new generation aimed at improved ruggedness, reliability and ease-of-use.

## Integrator Capabilities

The integrator algorithms include the following key capabilities:

- an autointegrate capability used to set up initial integrator parameters,

- the ability to define individual integration event tables for each chromatographic/electropherographic signal if multiple signals or more than one detector is used,

- interactive definition of integration events that allows users to graphically select event times,

- graphical manual or rubber-band integration of chromatogram/electropherograms requiring human interpretation (these events may also be recorded in the method and used as part of the automated operation),

- display and printing of integration results, and

- the ability to integrate at least 1000 peaks per chromatogram/electropherograms.

- integrator parameter definitions to set or modify the basic integrator settings for area rejection, height rejection, peak width and slope sensitivity,

- baseline control parameters, such as force baseline, hold baseline, baseline at all valleys, baseline at the next valley, fit baseline backwards from the end of the current peak,

- area summation control,

- negative peak recognition,

- tangent skim processing including solvent peak definition commands, and

- the ability to define individual front/tail tangent skim calculations event tables for all chromatographic/electropherographic signal,

- the ability of baseline correction parameters (non signal related),

- integrator control commands defining retention/migration time ranges for the integrator operation.

- peak shoulder allocation through the use of second derivative or degree of curvature calculations,

- improved sampling of non-equidistant data points for better performance with DAD LC data files that are reconstructed from DAD spectra.

# Overview

To integrate a chromatogram/electropherogram the integrator:

**1** defines the initial baseline,

**2** continuously tracks and updates the baseline,

**3** identifies the start time for a peak and marks this point with a vertical tick mark,

**4** finds the apex of each peak and prints the retention/migration time,

**5** identifies the end time for the peak, and marks this point with a vertical tick mark,

**6** constructs a baseline, and

**7** calculates the area, height, and peak width for each peak.

This process is controlled by *integration events*. The most important events are *initial slope sensitivity, peak width, area reject* and *height reject*. The software allows you to set initial values for these and other events. The initial values take effect at the beginning of the chromatogram. In addition, the auto integration function provides a set of initial events that you can optimize further.

In most cases, the initial events will give good integration results for the entire chromatogram, but there may be times when you want more control over the progress of an integration.

The software allows you to control how an integration is performed by enabling you to program new integration events at appropriate times in the chromatogram.

For more information, see "Initial Events" on page 105.

## Defining the Initial Baseline

Because baseline conditions vary according to the application and detector hardware, the integrator uses parameters from both the method and the data file to optimize the baseline.

Before the integrator can integrate peaks, it must establish a *baseline point*. At the beginning of the analysis, the integrator establishes an initial baseline level by taking the first data point as a tentative baseline point. It then attempts to redefine this initial baseline point based on the average of the input signal. If the integrator does not obtain a redefined initial baseline point, it retains the first data point as a potential initial baseline point.

## Tracking the Baseline

The integrator samples the digital data at a rate determined by the initial peak width or by the calculated peak width, as the run progresses. It considers each data point as a potential baseline point.

The integrator determines a *baseline envelope* from the slope of the baseline, using a baseline-tracking algorithm in which the slope is determined by the first derivative and the curvature by the second derivative. The baseline envelope can be visualized as a cone, with its tip at the current data point. The upper and lower acceptance levels of the cone are:

- + upslope + curvature + baseline bias must be lower than the threshold level,

- - upslope - curvature + baseline bias must be more positive (i.e. less negative) than the threshold level.

As new data points are accepted, the cone moves forward until a break-out occurs.

To be accepted as a baseline point, a data point must satisfy the following conditions:

- it must lie within the defined baseline envelope,

- the curvature of the baseline at the data point (determine by the derivative filters), must be below a critical value, as determined by the current slope sensitivity setting.

The initial baseline point, established at the start of the analysis is then continuously reset, at a rate determined by the peak width, to the moving average of the data points that lie within the baseline envelope over a period determined by the peak width. The integrator tracks and periodically resets the baseline to compensate for drift, until a peak up-slope is detected.

# Allocating the Baseline

The integrator allocates the chromatographic/electropherographic baseline during the analysis at a frequency determined by the peak width value. When the integrator has sampled a certain number of data points, it resets the baseline from the initial baseline point to the current baseline point. The integrator resumes tracking the baseline over the next set of data points and resets the baseline again. This process continues until the integrator identifies the start of a peak
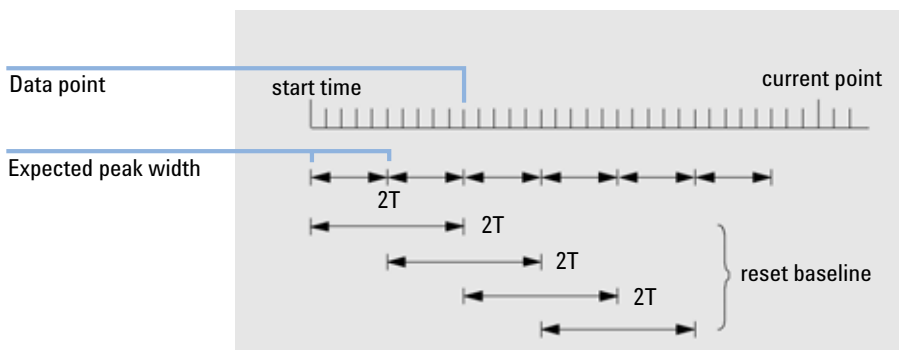


**Figure 4**     Baseline

At the start of the run, this baseline setting is used as the beginning baseline. If this is not set, the first data point is used. This baseline point is then periodically reset according to the following formula:

Areas are summed over a time T (expected peak width). This time can never be shorter than one data point. This continues as long as baseline condition exists. Slope and curvature are also taken. If both slope and curvature are less than the threshold, two summed areas are added together, and compared with the previous baseline. If the new value is less than the previous baseline, the new value immediately replaces the old one. If the new value is greater than the previous value, it is stored as a tentative new baseline value and is confirmed if one more value satisfies slope and curvature flatness criteria. This latter limitation is not in effect if negative peaks are allowed. During baseline, a check must also be made to examine fast rising solvents. They may be too fast for upslope detection. (By the time upslope is confirmed, solvent criterion may no longer be valid.) At first time through the first data point is baseline. It is replaced by the 2 T average if signal is on base. Baseline is then reset every T (see Figure 4 on page 75).

# Identifying the Cardinal Points of a Peak

The integrator determines that a peak may be starting when potential baseline points lie outside the baseline envelope, and the baseline curvature exceeds a certain value, as determined by the integrator's slope sensitivity parameter. If this condition continues, the integrator recognizes that it is on the up-slope of a peak, and the peak is processed.

### Start

**1** Slope and curvature within limit: continue tracking the baseline.

**2** Slope and curvature above limit: possibility of a peak.

**3** Slope remains above limit: peak recognized, cardinal point defined.

**4** Curvature becomes negative: front inflection point.

### Apex

**1** Slope passes through zero and becomes negative: apex of peak, cardinal point defined.

**2** Curvature becomes positive: rear inflection point.

### End

**1** Slope and curvature within limit: approaching end of the peak.

**2** Slope and curvature remain within limit: end of peak, cardinal point defined.

**3** The integrator returns to the baseline tracking mode.

# Definition of Terms

## Cardinal Points
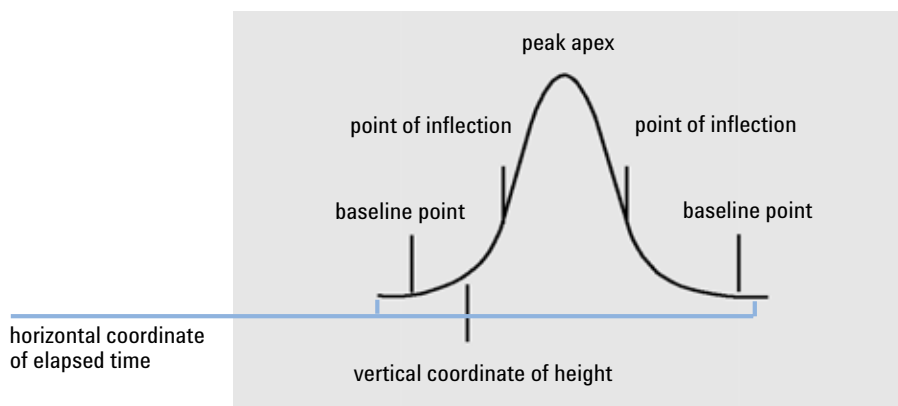


**Figure 5**    Cardinal points

Cardinal points are the points chosen by the integrator to define and quantify a peak. Baseline points, valley points, peak apex, and points of inflection are designated cardinal points and saved. Each cardinal point has a horizontal coordinate of elapsed time, a vertical coordinate of height from the baseline, and other parameters, such as peak type, separation codes, start/end values of potential peaks, and corresponding height, area and slope readings, that the integrator uses to calculate the peak areas.

## Solvent Peak

The solvent peak, which is generally a very large peak of no analytical importance, is not normally integrated. However, when small peaks of analytical interest elute close to the solvent peak, for example, on the tail of the solvent peak, special integration conditions can be set up to calculate their areas corrected for the contribution of the solvent peak tail.

## Shoulder (front, rear)

Shoulders occur when two peaks elute so close together that no valley exists between them, and they are unresolved. Shoulders may occur on the leading edge (front) of the peak, or on the trailing edge (rear) of the peak. When shoulders are detected, they may be integrated either by tangent skim or by drop-lines.

## Slope

The slope of a peak, which denotes the change of concentration of the component against time, is used to determine the onset of a peak, the peak apex, and the end of the peak.

# Principle of Operation
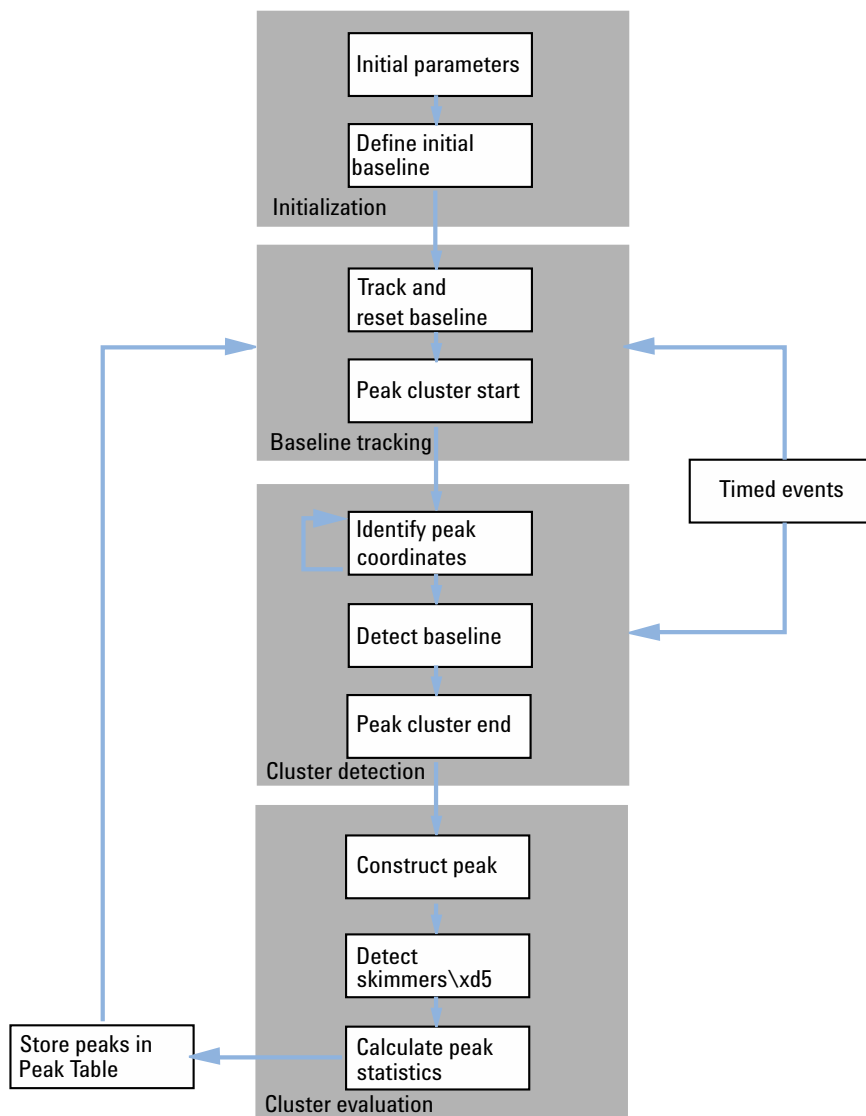


**Figure 6**     Integrator Flow Diagram

# Peak Recognition

The integrator uses several tools to recognize and characterize a peak:

- peak width,
- peak recognition filters,
- bunching,
- peak recognition algorithm,
- peak apex algorithm, and
- non-Gaussian calculations (for example tailing, merged peaks).

## Peak Width

During integration, the peak width is calculated from the peak area and height:

$Width = Area/Height$

or, if the inflection points are available, from the width between the inflection points.
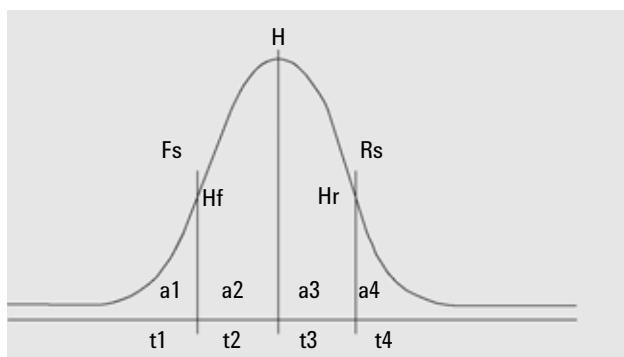


**Figure 7**     Peak width calculation

In Figure Peak Width Calculation, the total area, A, is the sum of the areas a1, a2, a3 and a4. Fs is the front slope at the inflection point, Rs is the rear slope at the inflection point. If either inflection point is not found, the peak width is defined as:

*Width = Adjusted area / Adjusted height*

The peak width setting controls the ability of the integrator to distinguish peaks from baseline noise. To obtain good performance, the peak width must be set close to the width of the actual chromatographic/electropherographic peaks.

There are three ways the peak width is changed:

- before the run, you can specify the initial peak width,
- during the run, the integrator automatically updates the peak width as necessary to maintain a good match with the peak recognition filters,
- during the run, you can reset or modify the peak width using a time-programmed event.

For peak width definitions used by System Suitably calculations please see "Peak Width" on page 80.

## Peak Recognition Filters

The integrator has three peak recognition filters that it can use to recognize peaks by detecting changes in the slope and curvature within a set of contiguous data points. These filters contain the first derivative (to measure slope) and the second derivative (to measure curvature) of the data points being examined by the integrator. The recognition filters are:

**Filter 1**    Slope (curvature) of two (three) contiguous data points

**Filter 2**    Slope of four contiguous data points and curvature of three non-contiguous data points

**Filter 3**    Slope of eight contiguous data points and curvature of three non-contiguous data points

The actual filter used is determined by the peak width setting. For example, at the start of an analysis, Filter 1 may be used. If the peak width increases during the analysis, the filter is changed first to Filter 2 and then to Filter 3.

To obtain good performance from the recognition filters, the peak width must be set close to the width of the actual chromatographic/electropherographic peaks. During the run, the integrator updates the peak width as necessary to optimize the integration.

The integrator calculates the updated peak width in different ways, depending on the instrument configuration:

For LC/CE configurations, the default peak width calculation uses a composite calculation:

*0.3 x (Right Inflection Point - Left Inflection point) + 0.7 x Area/Height*

For GC configurations, the default peak width calculation uses area/height. This calculation does not overestimate the width when peaks are merged above the half-height point.

In certain types of analysis, for example isothermal GC and isocratic LC analyses, peaks become significantly broader as the analysis progresses. To compensate for this, the integrator automatically updates the peak width as the peaks broaden during the analysis. It does this automatically unless the updating has been disabled or the peak width has been set to a specific value with a timed event.

The peak width update is weighted in the following way:

*0.75 x (existing peak width) + 0.25 x (width of current peak)*

If a timed integration event disables or sets the peak width to a specific value, the automatic peak width adjustment is disabled.

## Bunching

Bunching is the means by which the integrator keeps broadening peaks within the effective range of the peak recognition filters to maintain good selectivity.

The integrator cannot continue indefinitely to increase the peak width for broadening peaks. Eventually, the peaks would become so broad that they could not be seen by the peak recognition filters. To overcome this limitation, the integrator bunches the data points together, effectively narrowing the peak while maintaining the same area.

When data is bunched, the data points are bunched as two raised to the bunching power, i.e. unbunched = 1x, bunched once = 2x, bunched twice = 4x etc.

Bunching is based on the data rate and the peak width. The integrator uses these parameters to set the bunching factor to give the appropriate number of data points Table 8 on page 83.

Bunching is performed in the powers of two based on the expected or experienced peak width. The bunching algorithm is summarized in Table 8 on page 83.

**Table 8**     Bunching Criteria

| Expected Peak Width | Filter(s) Used | Bunching Done |
|---|---|---|
| 0 - 10 data points | First | None |
| 8 - 16 data points | Second | None |
| 12 - 24 data points | Third | None |
| 16 - 32 data points | Second | Once |
| 24 - 48 data points | Third | Once |
| 32 - 96 data points | Third, second | Twice |
| 64 - 192 data points | Third, second | Three times |

## The Peak Recognition Algorithm

The integrator identifies the start of the peak with a baseline point determined by the peak recognition algorithm. The peak recognition algorithm first compares the outputs of the peak recognition filters with the value of the initial slope sensitivity, to increase or decrease the up-slope accumulator. The integrator declares the point at which the value of the up-slope accumulator is $\geq 15$ the point that indicate that a peak has begun.

The peak recognition algorithm is shown in Figure 8 on page 84.
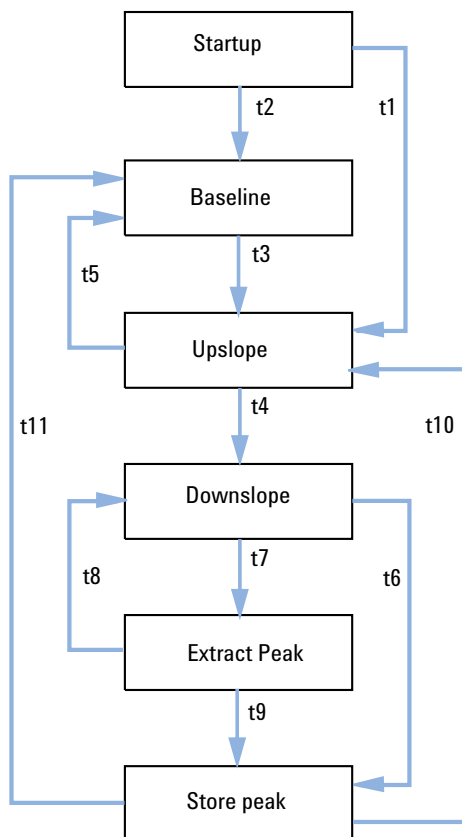
**Figure 8** Peak recognition

## The criteria are as follows:

**t1** Upslope counter is greater than or equal to 1

**t2** Upslope counter equals zero

**t3** Upslope counter is greater than or equal to 2

**t4** • Peak top found and half peak width found or
   • Peak top found and downslope counter is greater than or equal to 2

**t5** • Peak abort or
   • Baseline reset now

**t6**   • Peak valley found and upslope counter is greater than or equal to 2 or

 • Downslope sigma is greater than twice peak end sigma or

 • Baseline reset now or

 • Baseline reset next valley and peak valley found

**t7**   Downslope criterion is no longer met

**t8**   Downslope criterion is met again

**t9**   • Peak valley found and upslope counter is greater than or equal to 2 or

 • Downslope counter equals zero or

 • Downslope sigma is greater than peak end sigma or

 • Baseline reset now or

 • Baseline reset next valley

**t10**   Upslope counter is greater than or equal to 2

**t11**   Upslope counter is less than or equal to 1

### Peak Start

In Table 9 on page 85 the expected peak width determines which filter's slope and curvature values are compared with the Slope Sensitivity. For example, when the expected peak width is small, Filter 1 numbers are added to the up-slope accumulator. If the expected peak width increases, then the numbers for Filter 2 and, eventually, Filter 3 are used.

When the value of the up-slope accumulator is $\geq 15$, the algorithm recognizes that a peak may be starting.

**Table 9**   Incremental Values to Upslope Accumulator

| Derivative Filter 1 - 3 Outputs against Slope Sensitivity | Filter 1 | Filter 2 | Filter 3 |
|---|---|---|---|
| Slope > Slope Sensitivity | +8 | +5 | +3 |
| Curvature > Slope Sensitivity | +0 | +2 | +1 |
| Slope < (-) Slope Sensitivity | -8 | -5 | -3 |
| Slope > \|Slope Sensitivity\| | -4 | -2 | -1 |
| Curvature < (-) Slope Sensitivity | -0 | -2 | -1 |

### Peak End

In Table 10 on page 86the expected peak width determines which filter's slope and curvature values are compared with the Slope Sensitivity. For example, when the expected peak width is small, Filter 1 numbers are added to the down-slope accumulator. If the expected peak width increases, then the numbers for Filter 2 and, eventually, Filter 3 are used.

When the value of the down-slope accumulator is $\geq 15$, the algorithm recognizes that a peak may be ending.

**Table 10** Incremental Values for Downslope Accumulator

| Derivative Filter 1 - 3 Outputs against Slope Sensitivity | Filter 1 | Filter 2 | Filter 3 |
|---|---|---|---|
| Slope > Slope Sensitivity | +8 | +5 | +3 |
| Curvature > Slope Sensitivity | +0 | +2 | +1 |
| Slope < (-) Slope Sensitivity | -11 | -7 | -4 |
| Slope > \|Slope Sensitivity\| | -28 | -18 | -11 |
| Curvature < (-) Slope Sensitivity | -0 | -2 | -1 |

### The Peak Apex Algorithm

The peak apex is recognized as the highest point in the chromatogram by constructing a parabolic fit that passes through the highest data points.

## Non-Gaussian Calculations

### Merged Peaks

Merged peaks occur when a new peak begins before the end of peak is found. The figure illustrates how the integrator deals with merged peaks.
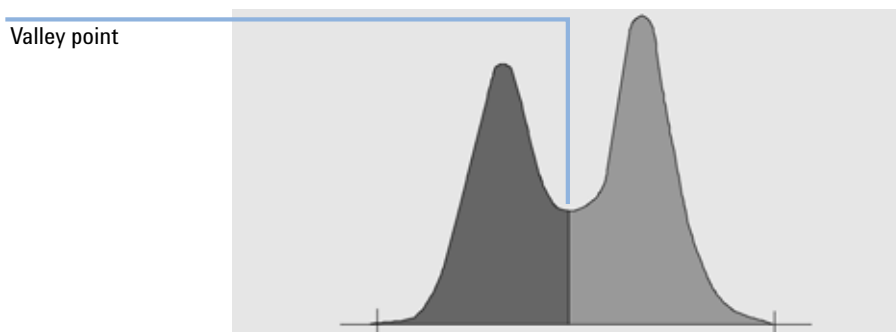
Valley point

**Figure 9** Merged Peaks

The integrator processes merged peaks in the following way:

**1** it sums the area of the first peak until the valley point.

**2** at the valley point, area summation for the first peak ends and summation for the second peak begins.

**3** when the integrator locates the end of the second peak, the area summation stops. This process can be visualized as separating the merged peaks by dropping a perpendicular from the valley point between the two peaks.

## Shoulders

Shoulders are unresolved peaks on the leading or trailing edge of a larger peak. When a shoulder is present, there is no true valley in the sense of negative slope followed by positive slope. A peak can have any number of front and/or rear shoulders.

**Figure 10**    Peak Shoulders

Shoulders are detected from the curvature of the peak as given by the second derivative. When the curvature goes to zero, the integrator identifies a point of inflection, such as points a and b in Figure 10 on page 88.
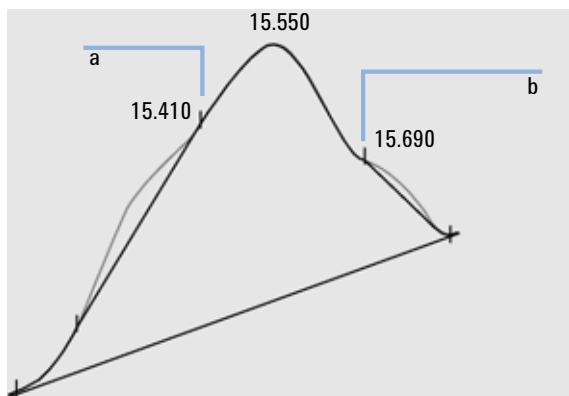
- A potential front shoulder exists when a second inflection point is detected before the peak apex. If a shoulder is confirmed, the start of the shoulder point is set at the maximum positive curvature point before the point of inflection.

- A potential rear shoulder exists when a second inflection point is detected before the peak end or valley. If a shoulder is confirmed, the start of the shoulder point is set at the target point from starting point to curve.

retention/migration time is determined from the shoulder's point of maximum negative curvature. With a programmed integration event, the integrator can also calculate shoulder areas as normal peaks with drop-lines at the shoulder peak points of inflection.

The area of the shoulder is subtracted from the main peak.

Peak shoulders can be treated as normal peaks by use of an integrator timed event.

# Baseline Allocation

After any peak cluster is complete, and the baseline is found, the integrator requests the baseline allocation algorithm to allocate the baseline using a pegs-and-thread technique. It uses trapezoidal area and proportional height corrections to normalize and maintain the lowest possible baseline. Inputs to the baseline allocation algorithm also include parameters from the method and data files that identify the detector and the application, which the integrator uses to optimize its calculations.

## Default Baseline Construction

In the simplest case, the integrator constructs the baseline as a series of straight line segments between:

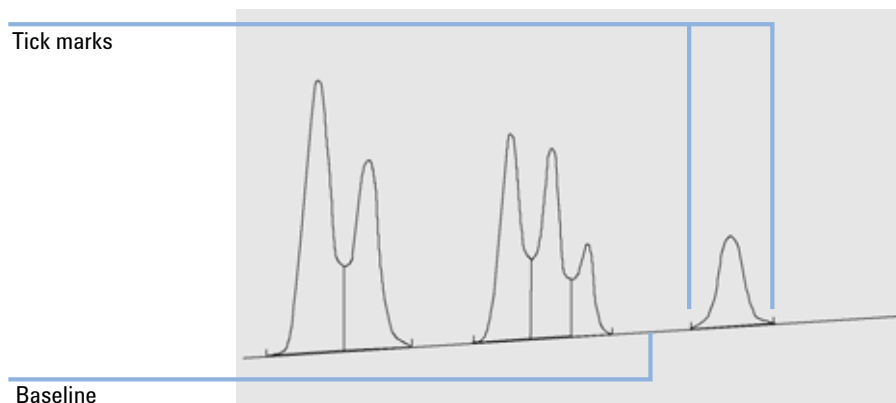- the start of baseline,
- the tick marks,
- the end of peak

Tick marks

Baseline

**Figure 11**    Default Baseline Construction

## The Start of the Baseline

If no baseline is found at the start of the run, the start of the baseline is established in one of the following ways:

- from the start of the run to the first baseline point, if the start of run point is lower than the first baseline point,
- from the start of the run to the first valley point, if the start of run point is lower than the first valley,
- from the start of the run to the first valley point, if the first valley penetrates an imaginary line drawn from the start of run to the first baseline,
- from the start of the run to a horizontal baseline extended to the first baseline point.

## Tick Marks

Tick marks identify the beginning and end of a peak. Their positions are determined by the peak start and peak end times, saved in the peak table.

## The End of the Baseline

The last valid baseline point is used to designate the end of the baseline. In cases where the run does not end on the baseline, the end of the baseline is calculated from the last valid baseline point to the established baseline drift.

If a peak ends in an apparent valley but the following peak is below the area reject value as you have set it, the baseline is projected from the beginning of the peak to the next true baseline point. If a peak starts in a similar way, the same rule applies.

# Baseline Penetration

A penetration occurs when the signal drops below the constructed baseline (point a in Figure 12 on page 91). If a baseline penetration occurs, that part of the baseline is generally reconstructed, as shown by points b in Figure 12 on page 91.
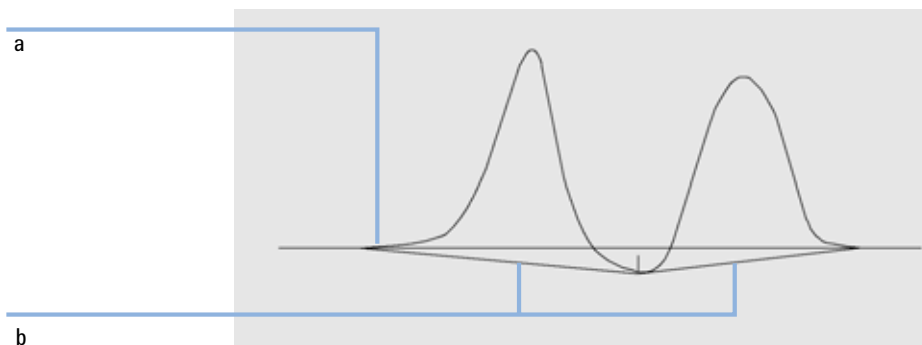


**Figure 12**    Baseline Penetration

You can use the following tracking options to remove all baseline penetrations:

## Classical Baseline Tracking (no penetrations)

When this option is selected, each peak cluster is searched for baseline penetrations. If penetrations are found, the start and/or end points of the peak are shifted until there are no penetrations left (compare the baselines in Figure 12 on page 91 and Figure 13 on page 92).
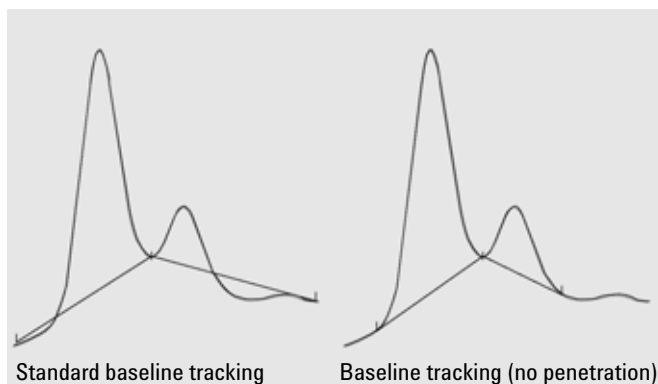
Standard baseline tracking          Baseline tracking (no penetration)

**Figure 13**     Standard baseline tracking and baseline tracking (no penetration)

| NOTE | Baseline tracking (no penetration) is not available for solvent peaks, with their child peaks and shoulders. |
|------|-------------|

### Advanced Baseline Tracking

In the advanced baseline tracking mode, the integrator tries to optimize the start and end locations of the peaks, re-establishes the baseline for a cluster of peaks, and removes baseline penetrations (see Figure 12 on page 91). In many cases, advanced baseline tracking mode gives a more stable baseline, which is less dependant on slope sensitivity.

## Peak Valley Ratio

This user-specified parameter is a constituent of advanced baseline tracking mode. It is used to decide whether two peaks that do not show baseline separation are separated using a drop line or a valley baseline. The integrator calculates the ratio between the baseline-corrected height of the smaller peak and the baseline-corrected height of the valley. When the peak valley ratio is lower than the user-specified value, a drop-line is used; otherwise, a baseline is drawn from the baseline at the start of the first peak to the valley, and from the valley to the baseline at the end of the second peak (compare Figure 13 on page 92 with Figure 14 on page 93).
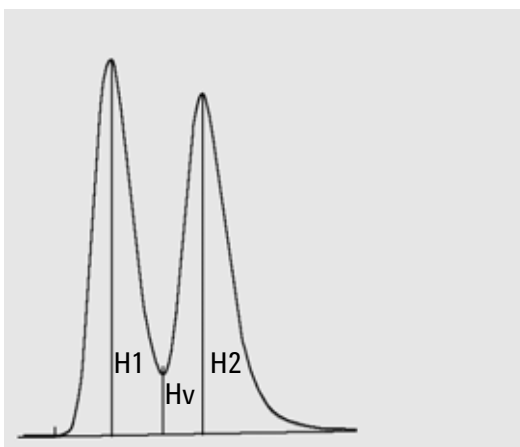
**Figure 14**    Peak Valley Ratio

The peak valley ratio is calculated using the following equations:

*H1 ≥ H2, Peak valley ratio = H2/Hv*

and

*H1 < H2, Peak valley ratio = H1/Hv*

shows how the user-specified value of the peak valley ratio affects the baselines.
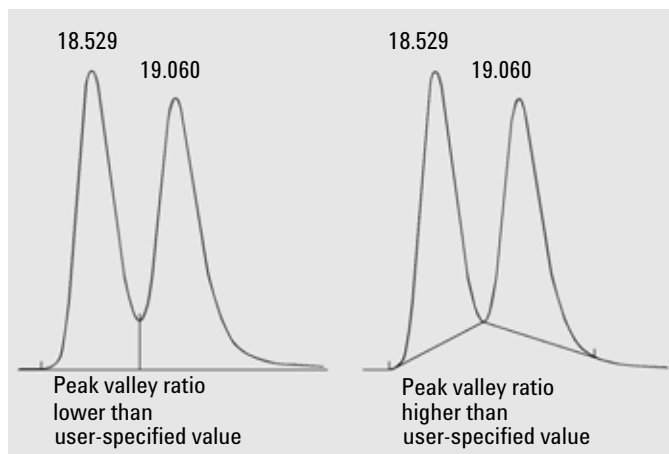


**Figure 15**    Effect of peak valley ratio on the baselines

# Tangent Skimming

Tangent skimming is a form of baseline constructed for peaks found on the upslope or downslope of a peak. When tangent skimming is enabled, four models are available to calculate suitable peak areas:

- exponential curve fitting,
- new exponential skim
- straight line skim,
- combined exponential and straight line calculations for the best fit (standard skims).

## Exponential Curve Fitting

This skim model draws a curve using an exponential equation through the start and end of the child peak (the height of the start of the child peak is corrected for the parent peak slope). The curve passes under each child peak that follows the parent peak; the area under the skim curve is subtracted from the child peaks and added to the parent peak (see Figure 17 on page 95).



14.296  14.357

14.144

**Figure 16** Exponential skim

## New Mode Exponential Curve Fitting

This skim model draws a curve using an exponential equation to approximate the leading or trailing edge of the parent peak. The curve passes under one or more peaks that follow the parent peak (child peaks). The area under the skim curve is subtracted from the child peaks and added to the main peak. More than one child peak can be skimmed using the same exponential model; all

peaks after the first child peak are separated by drop lines, beginning at the end of the first child peak, and are dropped only to the skim (see Figure 17 on page 95).

Parent peak

Child peaks

Drop lines

Exponential skim curve

Baseline of parent peak

14.144

14.296    14.357

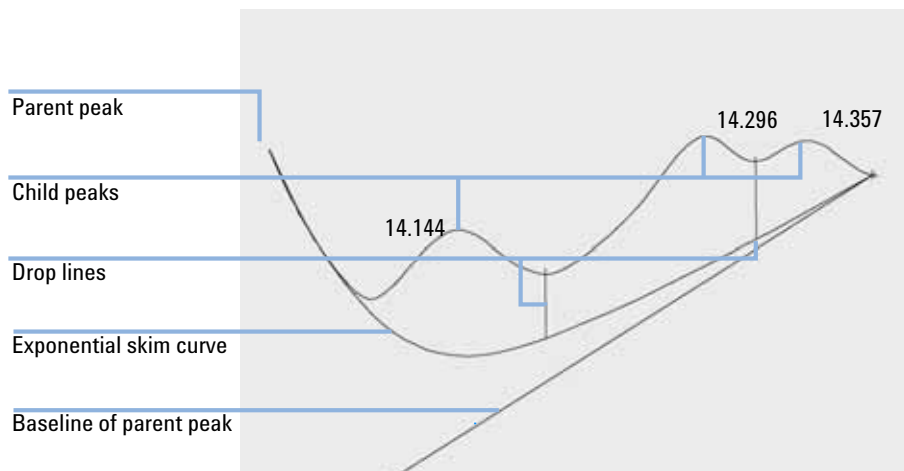**Figure 17**    New mode exponential skim

## Straight Line Skim

This skim model draws a straight line through the start and end of a child peak. The height of the start of the child peak is corrected for the parent peak slope. The area under the straight line is subtracted from the child peak and added to the parent peak (see Figure 18 on page 95).
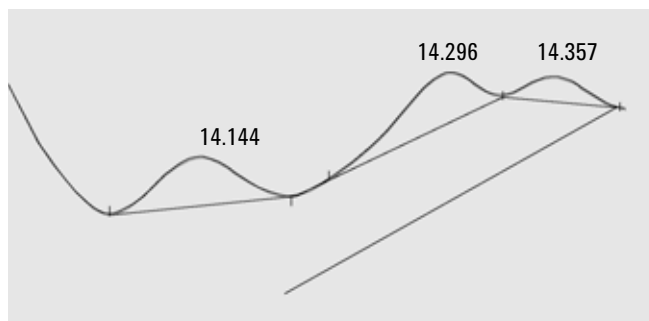
14.144

14.296    14.357

**Figure 18**    Straight line skim

## Standard Skims

The appropriate calculation is chosen for a particular application; by default, the chosen method is a combination of exponential and straight line calculations for the best fit.

The switch from an exponential to a linear calculation is performed in a way that eliminates abrupt discontinuities of heights or areas.

- When the signal is well above the baseline, the tail-fitting calculation is exponential.
- When the signal is within the baseline envelope, the tail fitting calculation is a straight line.

The combination calculations are reported as exponential or tangent skim.

## Skim Criteria

Two criteria determine whether a skim line is used to calculate the area of a child peak eluting on the trailing edge of a parent peak:

- tail skim height ratio
- valley height ratio

These criteria are not used if a timed event for an exponential is in effect, or if the parent peak is itself a child peak. The separation code between parent peak and child peak must be of type **Valley**.

**Tail Skim Height Ratio** is the ratio of the baseline-corrected height of the parent peak (Hp in Figure 19 on page 96) to the baseline-corrected height of the child peak (Hc). This ratio must be greater than the specified value for the child peak to be skimmed.
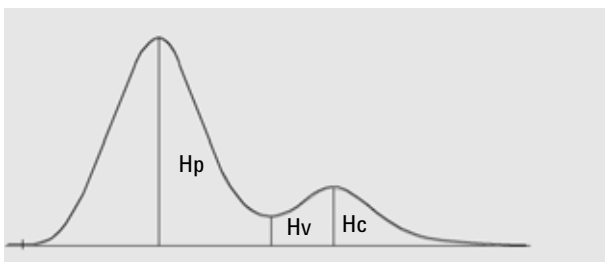


**Figure 19**    Skim criteria

You can disable exponential skimming throughout the run by setting the value of the tail skim height ratio to a high value or to zero.

**Valley Height Ratio** is the ratio of the height of the child peak above the baseline (Hc in Figure 19 on page 96) to the height of the valley above the baseline (Hv in same figure). This ratio must be smaller than the specified value for the child peak to be skimmed.

### Calculation of Exponential Curve Fitting for Skims

The following equation is used to calculate an exponential skim (refer to Figure 22 on page 98):

$$Hb = Ho \times exp\,(\text{-}\,B \times (Tr\,\text{-}\,To)) + A \times Tr + C$$

where

Hb = height of the exponential skim at time Tr

Ho = height (above baseline) of the start of the exponential skim

B = decay factor of the exponential function

To = time corresponding to the start of the exponential skim

A = slope of the baseline of the parent peak

C = offset of the baseline of the parent peak
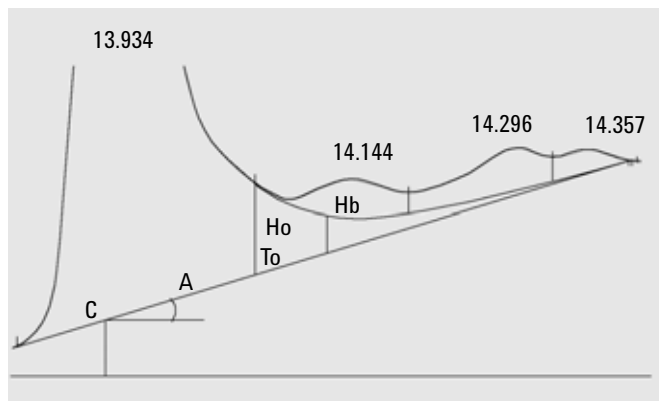


**Figure 20**    Values used to calculate an exponential skim

The exponential model is fitted through the part of the tail of the parent peak immediately before the first child peak. Figure 21 on page 98 shows the corrected curve of a child peak after tangent skimming.

Corrected child
peak curve
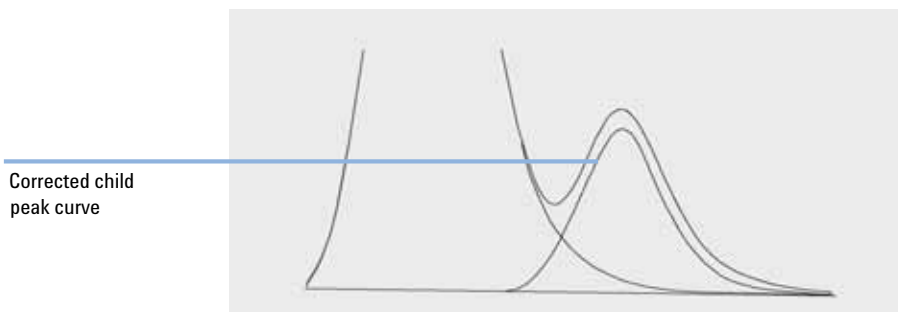
**Figure 21**    Tail-corrected child peak

## Front Peak Skimming

As for child peaks on the tail of a parent peak, special integration is required for some peaks on the front/upslope of a peak, see Figure 22 on page 98.
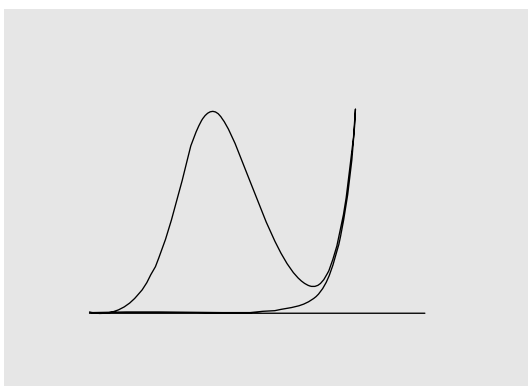
**Figure 22**    Front peak skimming

Front peak skimmimg is treated the same way as tail peak skimming, using the same skim models.

The skim criteria are:

- front skim height ratio
- valley height ratio

The valley height ratio takes the same value for both front peak skimming and tail peak skimming (see "Valley height ratio"); the front skim height ratio is calculated in the same way as the tail skim height ratio (see "Tail skim height ratio"), but can have a different value.

# Unassigned Peaks

With some baseline constructions, there are small areas that are above the baseline and below the signal, but are not part of any recognized peaks. Normally, such areas are neither measured nor reported. If unassigned peaks is turned on, these areas are measured and reported as unassigned peaks. The retention/migration time for such an area is the midpoint between the start and end of the area, as shown in Figure 23 on page 99.
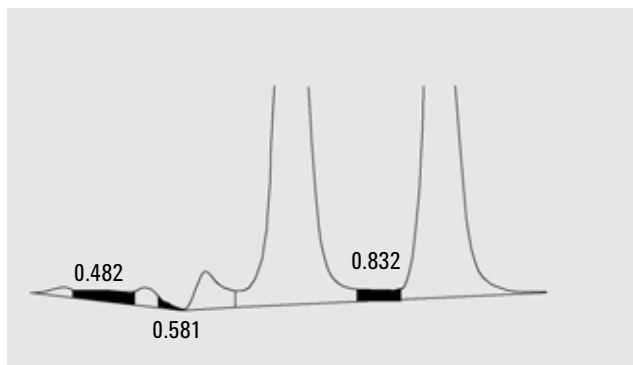


**Figure 23**     Unassigned Peaks

# Peak Separation Codes

In reports, each peak is assigned a two-, three- or four-character code that describes how the signal baseline was drawn.

## Characters 1 and 2

The first character describes the baseline at the start of the peak and the second character describes the baseline at the end of the peak.

**B**   The peak started or stopped on the baseline.

**V**   The peak started or stopped with a valley drop-line.

**P**   The peak started or stopped while the baseline was penetrated.

**H**   The peak started or stopped on a forced horizontal baseline.

**F**   The peak started or stopped on a forced point.

**M**   The peak was manually integrated.

**U**   The peak was unassigned.

Additional flags may also be appended (in order of precedence):

### Character 3

**D**   The peak was distorted.

**A**   The integration was aborted.

**U**   An under-range condition occurred.

**O**   An over-range condition occurred.

### Character 4

The fourth character describes the peak type:

**S**   The peak is a solvent peak.

**N**   The peak is a negative peak.

**+**   The peak is an area summed peak.

**T**   Tangent-skimmed peak (standard skim).

**X**   Tangent-skimmed peak (old mode exponential skim).

**E**   Tangent-skimmed peak (new mode exponential skim).

**m**   Peak defined by manual baseline.

**n**   Negative peak defined by manual baseline.

**t**   Tangent-skimmed peak defined by manual baseline.

**R**   The peak is a recalculated solvent peak.

**f**    Peak defined by a front shoulder tangent.

**b**    Peak defined by a rear shoulder tangent.

**F**    Peak defined by a front shoulder drop-line.

**B**    Peak defined by a rear shoulder drop-line.

**U**    The peak is unassigned.

# Peak Area Measurement

The final step in peak integration is determining the final area of the peak.

Peak areas are calculated from the contents of the cardinal point file. Cardinal points are the points chosen by the integrator to define and quantify a peak (see "Identifying the Cardinal Points of a Peak" on page 76). These include baseline points, valley points, peak apex, points at peak half height. Cardinal points have a horizontal coordinate of elapsed time, a vertical coordinate of height from the baseline, area, and other parameters that the integrator uses to calculate the peak areas.
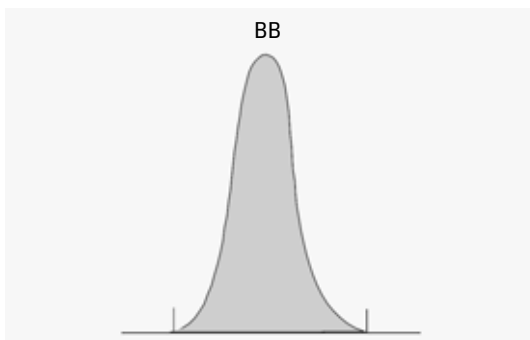


**Figure 24**     Area measurement for Baseline-to-Baseline Peaks

In the case of a simple, isolated peak, the peak area is determined by the accumulated area above the baseline between peak start and stop (identified by tick marks).

## Determination of the area

The area that the integrator calculates during integration is determined as follows:

- for baseline-to-baseline (BB) peaks, the area above the baseline between the tick marks, as in Figure 24 on page 102,

- for valley-to-valley (VV) peaks, the area above the baseline, segmented with vertical dropped lines from tick marks, as in Figure 25 on page 103,
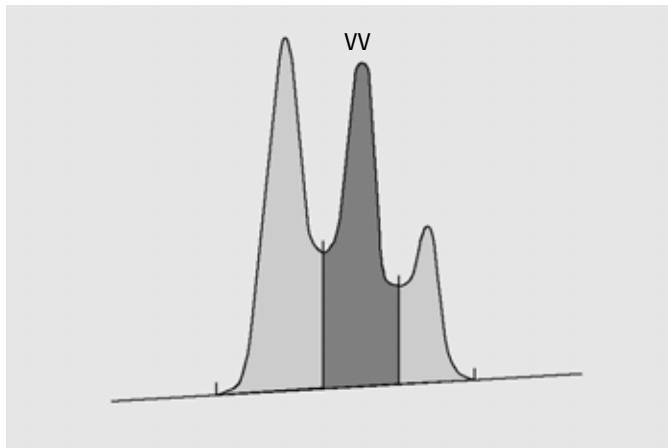


**Figure 25**    Area Measurement for Valley-to-Valley Peaks

- for tangent (T) peaks, the area above the reset baseline,
- for solvent (S) peaks, the area above the horizontal extension from the last-found baseline point and below the reset baseline given to tangent (T) peaks. A solvent peak may rise too slowly to be recognized, or there may be a group of peaks well into the run which you feel should be treated as a solvent with a set of riders. This usually involves a merged group of peaks where the first one is far larger than the rest. The simple drop-line treatment would exaggerate the later peaks because they are actually sitting on the tail of the first one. By forcing the first peak to be recognized as a solvent, the rest of the group is skimmed off the tail,
- negative peaks that occur below the baseline have a positive area, as shown in Figure 26 on page 104.

Total zone area - Area below baseline = Baseline corrected area
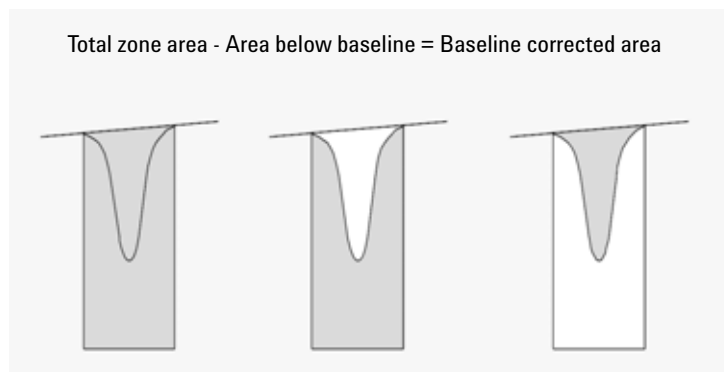
**Figure 26**    Area Measurement for Negative Peaks

## Units and Conversion Factors

Externally, the data contains a set of data points; they can be either sampled data or integrated data. In the case of integrated data, each data point corresponds to an area, which is expressed as $Height \times Time$. In the case of sampled data, each data point corresponds to a height.

Therefore, in the case of integrated data, height is a calculated entity, obtained by dividing area by the time elapsed since the preceding data point. In the case of sampled data, area is calculated by multiplying the data by the time elapsed since the preceding data point.

The integration calculation makes use of both entities. The units carried internally inside the integrator are: $counts \times milliseconds$ for area and $counts$ for height. This is done to provide a common base for integer truncations when needed. The measurements of time, area and height are reported in real physical units, irrespective of how they are measured, calculated and stored in the software.

# Integration Events

The integrator provides you with a number of initial and timed integrator events. Many events are on/off or start/stop pairs.

## Initial Events

**Initial Peak Width**  **Initial peak width** sets the integrator's internal peak width to this value for the start of run. This initial peak width is used to scale the accumulator that detects peak up-slope, down-slope, and tailing. The integrator updates the peak width when necessary during the run to optimize the integration. You specify the peak width in units of time that correspond to the peak width at half-height of the first expected peak (excluding the solvent peak).

**Slope Sensitivity**  **Slope sensitivity** is the setting for peak sensitivity. This is a setting that changes on a linear scale.

**Height reject**  **Height reject** sets peak rejection by final height. Any peaks that have heights less than the minimum height are not reported.

**Area reject**  **Area reject** sets peak rejection by final area. Any peaks that have areas less than the minimum area are not reported.

**Shoulder detection**  When **shoulder detection** is on, the integrator detects shoulders using the curvature of the peak as given by the second derivative. When the curvature goes to zero, the integrator identifies this point of inflection as a possible shoulder. If the integrator identifies another point of inflection before the apex of the peak, a shoulder has been detected.

### Peak Width

The peak width setting controls the selectivity of the integrator to distinguish peaks from baseline noise. To obtain good performance, the peak width must be set close to the width at half-height of the actual chromatographic/electropherographic peaks. The integrator updates the peak width when necessary during the run to optimize the integration.

## Choosing Peak Width

Choose the setting that provides just enough filtering to prevent noise being interpreted as peaks without distorting the information in the signal.

- To choose a suitable initial peak width for a single peak of interest, use the peak's time width as the base as a reference.
- To choose a suitable initial peak width when there are multiple peaks of interest, set the initial peak width to a value equal to or less than the narrowest peak width to obtain optimal peak selectivity.

If the selected initial peak width is too low, noise may be interpreted as peaks. If broad and narrow peaks are mixed, you may decide to use runtime programmed events to adjust the peak width for certain peaks. Sometimes, peaks become significantly broader as the analysis progresses, for example in isothermal GC and isocratic LC analyses. To compensate for this, the integrator automatically updates the peak width as peaks broaden during an analysis unless disabled or set with a timed event.

The Peak Width update is weighted in the following way:

*0,75 × (existing peak width) + 0,25 × (width of current peak)*

If a timed integration event disables or sets the peak width to a specific value, the automatic peak width adjustment is disabled.

## Height Reject and Peak Width

Both **peak width** and **height reject** are very important in the integration process. You can achieve different results by changing these values.

- Increase both the height reject and peak width where relatively dominant components must be detected and quantified in a high-noise environment. An increased peak width improves the filtering of noise and an increased height reject ensures that random noise is ignored.
- Decrease height reject and peak width to detect and quantify trace components, those whose heights approach that of the noise itself. Decreasing peak width decreases signal filtering, while decreasing height reject ensures that small peaks are not rejected because they have insufficient height.
- When an analysis contains peaks with varying peak widths, set peak width for the narrower peaks and reduce height reject to ensure that the broad peaks are not ignored because of their reduced height.

## Tuning Integration

It is often useful to change the values for the slope sensitivity, peak width, height reject, and area reject to customize integration.

Figure 27 on page 107 shows how these parameters affect the integration of five peaks in a signal.
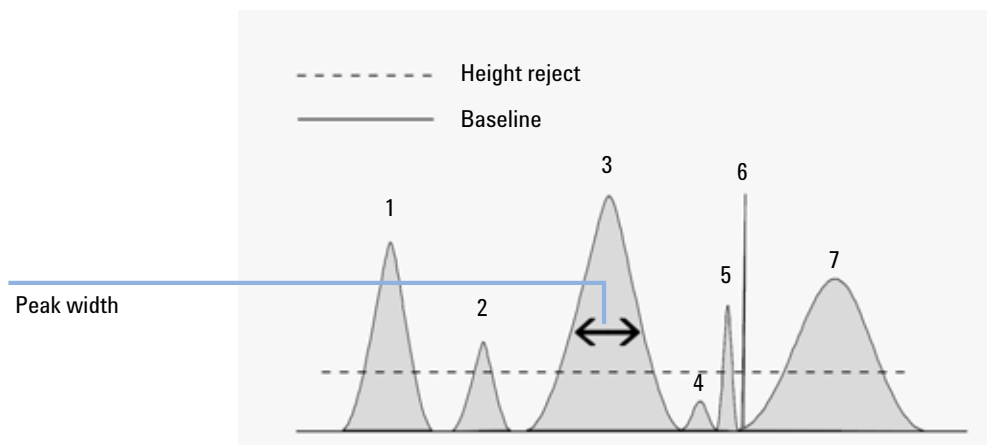


**Figure 27** Using Initial Events

A peak is integrated only when all of the four integration parameters are satisfied. Using the peak width for peak 3, the area reject and slope sensitivity shown in Figure 27 on page 107, only peaks 1, 3, 5 and 7 are integrated.

**Peak 1**     is integrated as all four integration parameters are satisfied.

**Peak 2**     is rejected because the area is below the set area reject value.

**Peak 3**     is integrated as all four integration parameters are satisfied.

**Peak 4**     is not integrated because the peak height is below the Height Reject.

**Peak 5**     is rejected because the area is below the set area reject value.

**Peak 6**     is not integrated; filtering and bunching make the peak invisible.

**Peak 7**     is integrated.

**Table 11** Height and Area Reject Values

| Integration Parameter | Peak 1 | Peak 2 | Peak 3 | Peak 4 | Peak 5 | Peak 7 |
|---|---|---|---|---|---|---|
| Height reject | Above | Above | Above | Below | Above | Above |
| Area reject | Above | Below | Above | Below | Below | Above |
| Peak integrated | Yes | No | Yes | No | No | Yes |

## Timed Events

You can use timed events to customize signal baseline construction when default construction is not appropriate. These events can be useful for summing final peak areas and for correcting short- and long-term baseline aberrations. For further information about integration events see "Initial Events" on page 105.

## Autointegrate

The **Autointegrate** function provides a starting point for setting initial events. This is particularly useful when you are implementing a new method. You start with a default integration events table that contains no timed events; you can then optimize the parameters proposed by the Autointegrate function for general use.

### Principles of Operation

The **Autointegrate** function reads the chromatogram data and calculates the optimal values for the initial integration parameters for each signal in the chromatogram object.

The algorithm examines 1% at the start and end of the chromatogram and determines the noise and slope for this part. Noise is determined as 3 times the standard deviation of the linear regression divided by the square root of the percent number of points used in the regression. These values are used to assign appropriate values to the height reject & slope sensitivity for the integration. The algorithm then assigns a temporary value for the peak width, depending on the length of the chromatogram, using 0.5% for LC and 0.3% to

0.2% for GC. The initial area reject is set to zero and a trial integration is performed. The trial is repeated several times if necessary, adjusting the parameters each time until at least 5 peaks are detected or integration is performed with an initial height reject of 0. The trial integration is terminated if the above conditions are not met after 10 trials.

The results of the integration are examined and the peak width is adjusted based on the peak widths of the detected peaks, biasing the calculation towards the initial peaks. The peak symmetry of the detected peaks is used to include only those peaks with symmetry between 0.8 and 1.3 for the peak width calculation. If not enough symmetric peaks are found, this limit is relaxed to *minSymmetry/1.5* and *maxSymmetry×1.5*. The baseline between the peaks is then examined to refine the earlier values of height reject & slope sensitivity. The area reject is set to 90% of the minimum area of the most symmetric peak detected during the trial integration.

The chromatogram is re-integrated using these final values for the integration parameters, and the results of the integration are stored.

### Autointegrate Parameters

The following parameters are set by the autointegrate function:

- Initial slope sensitivity
- Initial height
- Initial peak width
- Initial area reject

# Manual Integration

This type of integration allows you to integrate selected peaks or groups of peaks. Except for the initial area reject value, the software's event integration is ignored within the specified range of manual integration. If one or more of the peaks resulting from manual integration is below the area reject threshold, it is discarded. The manual integration events use absolute time values. They do not adjust for signal drift.

**Manual Integration** enables you to define the peak start and stop points, and then include the recalculated areas in quantification and reporting. Manually-integrated peaks are labeled in reports with the peak separation code M.

**Manual Integration** offers the following features:

Draw Baseline **Draw Baseline** specifies where the baselines are to be drawn for a peak or set of peaks. You can also specify whether peaks in the range given should be automatically separated at all valley points.

Negative Peaks **Negative Peaks** specifies when to treat any areas below the baseline as negative peaks. You can also specify whether peaks in the range given should be automatically separated at all valley points.

Tangent Skim **Tangent Skim** calculates the areas of peaks tangentially skimmed off a main peak. The area of the tangent skimmed peak is subtracted from the area of the main peak.

Split Peak **Split Peak** specifies a point where to split a peak with a drop-line.

Delete Peak(s) **Delete Peak(s)** deletes one or more peaks from the integration results.

## Peak Separation Codes for Manually-Integrated Peaks

Manually-integrated peaks are labeled in the integration reports by the peak code *MM*.

If there is a peak before the manually-integrated peak, and the end of this peak changes because of the manual integration, it is given the code *F* (forced).

A solvent peak which has been affected by manual integration, such as tangent skim, are labeled *R* (re-calculated solvent).

## Saving Manual Integration Events

As manual integration events are typically very specific for a certain data files, in ChemStation B.04.01 these events can be stored directly in the data file instead of the method. Each time the data file is reviewed or reprocessed, the manual events in the data file are automatically applied. A run containing manual integration events is marked in the **Navigation Table** in the corresponding column.

In addition to the tools for drawing a baseline and deleting a peak manually, three additional tools are available in the user interface to

- Save manual events of the currently shown chromatograms into the data file,
- Remove all events from the currently shown chromatograms,
- Undo the last manual integration events (available until the event is saved).

When continuing to the next data file during review in the **Navigation Table**, ChemStation will check for unsaved manual integration events and ask the user whether he wants to save the events.

Manual events stored in the data file during review in the **Navigation Table** do not interfere with manual integration events stored during review in the **Batch** mode. These two ways of review are completely separated with regard to the manual events of a data file.

In ChemStation revisions prior to B.04.01, manual integration events could only be stored in the method. In B.04.01, this workflow can still be used. The **Integration** menu in **Data Analysis** view provides the following items in order to handle manual integration events with the method:

- **Update Manual Events of Method**: Save newly drawn manual events to the method.
- **Apply Manual Events from Method**: Apply the manual events currently saved in the method to the currently loaded data file.
- **Remove Manual Events from Method**: Delete the manual events from the method.

In order to convert manual events stored in a method to storage in the data file, apply the events from the method and store the results in the data file. If wanted, remove the events from the method.
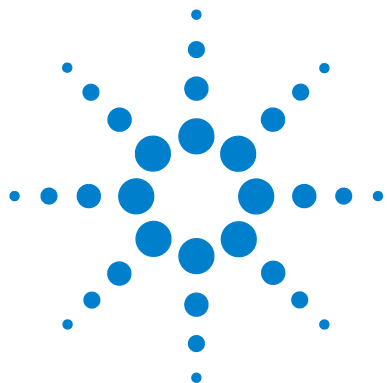
In case the **Manual Events** checkbox of the **Integration Events Table** of a method is enabled, the manual events of the method are always applied when loading a data file using this method. If the data file contains additional manual events, they are applied after the events of the method. When the **Manual Events** checkbox is enabled, the user is never asked to save the events to the data file.

### Conversion of Manual Integration Events Saved in a Method

In ChemStation revisions prior to B.04.01, manual integration events were stored in the method instead of the individual data file. In order to store the manual events in a data file specific way, it was most convenient to use the individual method DA.M of each specific data file.

The **Integration** menu in **Data Analysis** view provides the following items in order to either convert manual integration events in the method to events stored in the data file, or to continue to work with manual events stored in the method:

- **Update Manual Events of Method**: Save newly drawn manual events to the method.
- **Apply Manual Events from Method**: Apply the events currently saved in the method to the currently loaded data file.
- **Remove Manual Events from Method**: Delete the manual events from the method.

# 5

# Quantification

This chapter describes how ChemStation does quantification. It gives details on area% and height% calculations, external standard (ESTD) calculation, norm% calculation, internal standard (ISTD) calculation, and quantification of unidentified peaks.

**Agilent Technologies**

# What is Quantification?

After the peaks have been integrated and identified, the next step in the analysis is quantification. Quantification uses peak area or height to determine the concentration of a compound in a sample.

A quantitative analysis involves many steps which are briefly summarized as follows:

- Know the compound you are analyzing.

- Establish a method for analyzing samples containing this compound.

- Analyze a sample or samples containing a known concentration or concentrations of the compound to obtain the response due to that concentration.

    You may alternatively analyze a number of these samples with different concentrations of the compounds of interest if your detector has a non-linear response. This process is referred to as *multi-level calibration*.

- Analyze the sample containing an unknown concentration of the compound to obtain the response due to the unknown concentration.

- Compare the response of the unknown concentration to the response of the known concentration to determine how much of the compound is present.

To obtain a valid comparison for the unknown sample response to that of the known sample, the data must be acquired and processed under identical conditions.

# Quantification Calculations

The ChemStation offers the following calculation procedures for determining the concentration of each component present in a mixture:

- Percent
- Normalization
- External standard (ESTD)
- ESTD%
- Internal standard (ISTD)
- ISTD%

The calculations used to determine the concentration of a compound in an unknown sample depend on the type of quantification. Each calculation procedure uses the peak area or height for the calculation and produces a different type of report.

# Correction Factors

The quantification calculations use four correction factors, the *absolute response factor*, the *multiplier*, the *dilution factor*, and the *sample amount*. These factors are used in the calibration procedures to compensate for variations in detector response to different sample components, concentrations, sample dilutions, sample amounts, and for converting units.

## Absolute Response Factor

The absolute response factor for a sample component represents the amount of the component divided by the measured area or height of the component's peak in the analysis of a calibration mixture. The absolute response factor, which is used by each calibrated calculation procedure, corrects for detector response to individual sample components.

## Multiplier

The multiplier is used in each calculation formula to multiply the result for each component. The multiplier may be used to convert units to express amounts.

## Dilution Factor

The dilution factor is a number by which all calculated results are multiplied before the report is printed. You can use the dilution factor to change the scale of the results or correct for changes in sample composition during pre-analysis work. You can also use the dilution factor for any other purposes that require the use of a constant factor.

# Sample Amount

If the ESTD% or ISTD% calculations are selected, the ESTD and ISTD reports give relative values rather than absolute values, that is, the amount of each component is expressed as a percentage of the sample amount. The sample amount is used in ESTD% and ISTD% reports to convert the absolute amount of the components analyzed to relative values by dividing by the value specified.

# Uncalibrated Calculation Procedures

Uncalibrated calculation procedures do not require a calibration table.

## Area% and Height%

The Area% calculation procedure reports the area of each peak in the run as a percentage of the total area of all peaks in the run. Area% does not require prior calibration and does not depend upon the amount of sample injected within the limits of the detector. No response factors are used. If all components respond equally in the detector and are eluted, then Area% provides a suitable approximation of the relative amounts of components.

Area% is used routinely where qualitative results are of interest and to produce information to create the calibration table required for other calibration procedures.

The Height% calculation procedure reports the height of each peak in the run as a percentage of the total height of all peaks in the run.

# Calibrated Calculation Procedures

The external standard (ESTD), normalization, and internal standard (ISTD) calculation procedures require response factors and therefore use a calibration table. The calibration table specifies conversion of responses into the units you choose by the procedure you select.

# ESTD Calculation

The ESTD procedure is the basic quantification procedure in which both calibration and unknown samples are analyzed under the same conditions. The results from the unknown sample are then compared with those of the calibration sample to calculate the amount in the unknown.

The ESTD procedure uses absolute response factors unlike the ISTD procedure. The response factors are obtained from a calibration and then stored. In following sample runs, component amounts are calculated by applying these response factors to the measured sample amounts. Make sure that the sample injection size is reproducible from run to run, since there is no standard in the sample to correct for variations in injection size or sample preparation.

When preparing an ESTD report, the calculation of the amount of a particular compound in an unknown sample occurs in two steps:

**1** An equation for the curve through the calibration points for this compound is calculated using the type of fit specified in the Calibration Settings or Calibration Curve dialog box.

**2** The amount of the compound in the unknown is calculated using the equation described below. This amount may appear in the report or it may be used in additional calculations called for by Multiplier, Dilution Factor, or Sample Amount values before being reported.

If the ESTD report is selected, the equation used to compute the absolute amount of component x is:

$$\text{Absolute Amt of x} = Response_x \cdot RF_x \cdot M \cdot D$$

where:

*Responsex* is the response of peak x;

*RFx* is the response factor for component x, calculated as:

$$RF_x = \frac{\text{Amount}_x}{\text{Response}_x}$$

*M* is the multiplier.
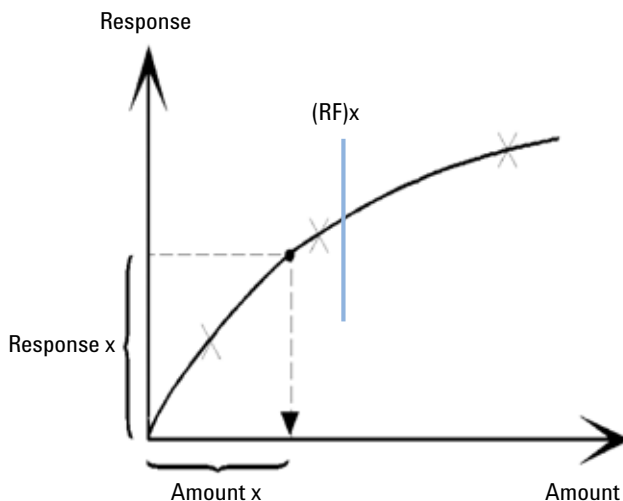
D is the dilution factor.



**Figure 28**    Response Factor

The multiplier and dilution factor are read either from the**Calibration Settings** or from the**Sample Information** dialog box.

If the ESTD% report is selected and sample amount is not zero, the relative amount (%) of a component x is calculated as shown below:

$$\text{Relative Amt of x} = \frac{\{\text{Absolute Amt of x}\} \cdot 100}{\text{Sample Amount}}$$

where:

*Absolute amount of x* is calculated as shown above in the ESTD calculation;

*Sample amount* is obtained from the Sample Information box, or from the Calibration Settings dialog box for single runs. If sample amount is zero, the ESTD is calculated.

# Norm% Calculation

In the normalization method, response factors are applied to the peak areas (or heights) to compensate for changes that occur in detector sensitivity for the different sample components.

The Norm% report is calculated in the same way as an ESTD report except that there is an additional step to calculate the relative rather than absolute amounts of compounds.

The Norm% report has the same disadvantage as the Area% and Height% reports. Any changes that affect the total peak area will affect the concentration calculation of each individual peak. The normalization report should only be used if all components of interest are eluted and integrated. Excluding selected peaks from a normalization report will change the reported results in the sample.

The equation used to calculate the Norm% of a component x is:

$$\text{Norm\% of x} = \frac{\text{Response}_x \cdot \text{RF}_x \cdot 100 \cdot M \cdot D}{\sum \{\text{Response} \cdot RF\}}$$

where:

| | |
|---|---|
| *Response*$_x$ | is the area (or height) of peak x, |
| *RF*$_x$ | is the response factor, |
| $\Sigma$ *(Response · RF)* | is the total of all the *(Response · RF)* products for all peaks including peak x, |
| *M* | is the multiplier, |
| *D* | is the dilution factor. |

The multiplier and dilution factor are read either from the **Calibration Settings**, or from the **Sample Information** dialog box.

# ISTD Calculation

The ISTD procedure eliminates the disadvantages of the ESTD method by adding a known amount of a component which serves as a normalizing factor. This component, the *internal standard*, is added to both calibration and unknown samples.

The software takes the appropriate response factors obtained from a previous calibration stored in the method. Using the internal standard concentration and peak areas or heights from the run, the software calculates component concentrations.

The compound used as an internal standard should be similar to the calibrated compound, both chemically and in retention/migration time, but it must be chromatographically distinguishable.

**Table 12** ISTD Procedure

| Advantages | Disadvantages |
| --- | --- |
| Sample-size variation is not critical. | The internal standard must be added to every sample. |
| Instrument drift is compensated by the internal standard. | |
| The effects of sample preparations are minimized if the chemical behavior of the ISTD and unknown are similar. | |

If the ISTD procedure is used for calibrations with a non-linear characteristic, care must be taken that errors which result from the calculation principle do not cause systematic errors. In multi-level calibrations, the amount of the ISTD compound should be kept constant, i.e. the same for all levels if the calibration curve of the compound is non-linear.

In the internal standard analysis, the amount of the component of interest is related to the amount of the internal standard component by the ratio of the responses of the two peaks.

In a two-run ISTD calibration, the calculation of the corrected amount ratio of a particular compound in an unknown sample occurs in the following stages:

# Run 1: Calibration

**1** The calibration points are constructed by calculating an amount ratio and a response ratio for each level of a particular peak in the calibration table.

The amount ratio is the amount of the compound divided by the amount of the internal standard at this level.

The response ratio is the area of the compound divided by the area or height of the internal standard at this level.

**2** An equation for the curve through the calibration points is calculated using the type of curve fit specified in the Calibration Settings dialog box or Calibration Curve dialog box.

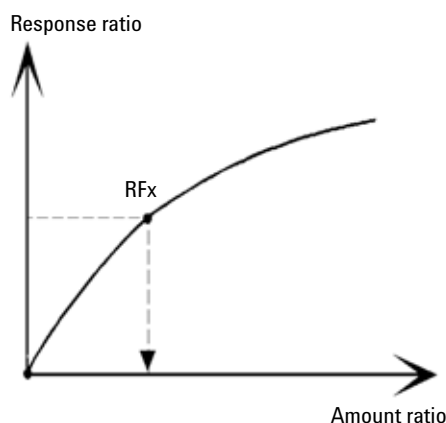$$RF_x = \frac{\text{Amount Ratio}}{\text{Response Ratio}}$$



**Figure 29** Amount Ratio

# Run 2: Unknown Sample

**1** The response of the compound in the unknown sample is divided by the response of the internal standard in the unknown sample to give a response ratio for the unknown.

**2** An amount ratio for the unknown is calculated using the curve fit equation determined in step 2 above, and the actual amount of ISTD in the sample.

## ISTD Calculation of Calibrated Peaks

The equations used to calculate the actual amount of a calibrated component x for a single-level calibration are:

$$\text{Response Ratio} = \frac{\text{Response}_x}{\text{Response}_{\text{ISTD}}}$$

$$\text{Actual Amount of x} = \text{RF}_x \cdot \{\text{Response Ratio}\}_x \cdot \text{Actual Amount of ISTD} \cdot M \cdot D$$

where:

$RF_x$ is the response factor for compound x;

The actual amount *(Actual Amt)* of ISTD is the value that was entered in the Calibration Settings dialog box or the Sample Info dialog box for the internal standard added to the unknown sample;

*M* is the multiplier.

D is the dilution factor.

If the ISTD% report type is selected, the following equation is used to calculate the relative (%) amount of component x:

$$\text{Relative Amt of x} = \frac{\{\text{Absolute Amt of x}\} \cdot 100}{\text{Sample Amount}}$$

## ISTD Calculation of Uncalibrated Peaks

There are two ways to define the response factor which is used to calculate the amount for unidentified peaks.

1 Use the fixed response factor set in the With Rsp Factor box of the Calibration Settings dialog box. You can choose to correct the fixed response factor by specifying an ISTD correction.

$$\text{Actual Amount of x} = \text{RF}_x \cdot \{\text{Response Ratio}\}_x \cdot \text{Actual Amount of ISTD} \cdot M \cdot D$$

$$\text{Response Ratio} = \frac{\text{Response}_x}{\text{Response}_{\text{ISTD}}}$$

$RF_x$ is the Response Factor set in the **Calibration Settings** dialog box.

You can see from these formulae that the variations in the ISTD response are used to correct the quantification of the unknown component.

**2** Use a calibrated peak. This ensures that the same response factor is used for the quantification of all peaks. The response factor of the selected compound and the uncalibrated peaks is corrected during all recalibrations. If the calibrated peak response factor changes, then the response factor for the unidentified peaks also changes by the same amount. If a Calibration Table is already set up, you can select a compound from the Using Compound combo box in the Calibration Settings dialog box.

The equations used to calculate the actual amount of an uncalibrated peak x are shown above.

# 6
# Peak Identification

This chapter describes the concepts of peak identification.

**Agilent Technologies**

# What is Peak Identification?

Peak identification identifies the components in an unknown sample based on their chromatographic/electropherographic characteristics determined by the analysis of a well-defined calibration sample.

The identification of these components is a necessary step in quantification if the analytical method requires quantification. The signal characteristics of each component of interest are stored in the calibration table of the method.

The function of the peak identification process is to compare each peak in the signal with the peaks stored in the calibration table.

The calibration table contains the expected retention/migration times of components of interest. A peak that matches the retention/migration time of a peak in the calibration table is given the attributes of that component, for example, the name and response factor. Peaks that do not match any of the peaks in the calibration table are classified as unknown. The process is controlled by:

- the retention/migration time in the calibration table for peaks designated as time reference peaks,
- the retention/migration time windows specified for reference peaks,
- the retention/migration times in the calibration table for the calibrated peaks that are not time reference peaks,
- the retention/migration time window specified for these non-reference peaks, and
- the presence of any additional qualifying peaks in the correct ratios.

# Peak Matching Rules

The following rules apply to the peak matching process:

- if a sample peak falls within the peak matching window of a component peak from the calibration table, the peak is given the attributes of that component,

- if more than one sample peak falls within the peak matching window, then, the peak closest to the expected retention/migration time is identified as that component,

- if a peak is a time reference or internal standard, then the largest peak in the window is identified as that component,

- if peak qualifiers are also used then the peak ratio is used in combination with the peak matching window to identify the component peak,

- if the peak is a qualifier peak, the measured peak closest to the main peak of the compound is identified, and

- if a sample peak does not fall in any peak matching window, it is listed as an unknown component.

# Types of Peak Identification

There are different techniques that can be used to match sample peaks with those in the calibration table of the ChemStation software.

## Absolute Retention/Migration Time

The retention/migration time of the sample peak is compared with the expected retention/migration time specified for each component in the calibration table.

## Corrected Retention/Migration Time

The expected retention/migration times of component peaks are corrected using the actual retention/migration times of one or more reference peaks, and the matching process is done using these corrected (relative) retention/migration times. The reference peak or peaks must be specified in the calibration table.

## Peak Qualifiers

In addition to identifying peaks by retention/migration time, you can use peaks qualifiers to allow a more precise result. If more than one peak occurs in a retention/migration time window then qualifiers should be used to identify the correct compound.

# Amount Limits

The amount limits defined in the Compound Details dialog box are used to qualify the peak identification. If the amount of the identified compound is inside the amount limits the peak identification is indicated in the report.

# Absolute Retention/Migration Time

A retention/migration time window is used in the peak matching process. The retention/migration time window is a window which is centered on the retention/migration time for an expected peak. Any sample peak that falls within this window may be considered as a candidate for component identification.

Figure 30 on page 132 shows a retention/migration time window for peak 2 which is between 1.809 and 2.631 minutes where the expected retention/migration time is 2.22 minutes. There are two possibilities for peak 2. One is at 1.85 minutes and the other at 2.33 minutes. If the expected peak is a non-reference peak, the peak closest to the expected retention/migration time of 2.22 minutes is selected.

If the expected peak is a time reference or internal standard, the largest peak in the window is selected.

In both cases the ChemStation selects the peak at 2.33 minutes. If the two peaks were the same size then the peak closest to the center of the window is chosen.
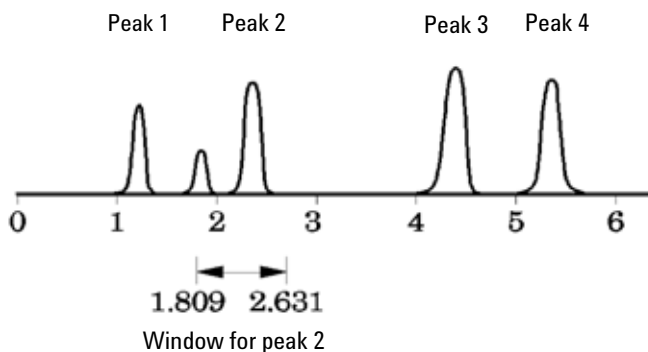


**Figure 30**    Retention/Migration Time Windows

Three types of windows are used when trying to locate peaks.

• reference peak windows which apply to reference peaks only,

• non-reference peak windows which apply to all other calibrated peaks, and

- specific window values for individual components which are set in the
  **Compound Details** dialog box.

The default values for these windows are entered in the Calibration Settings
dialog box. The width on either side of the retention/migration time that
defines the peak matching window is the sum of the absolute and percentage
windows.

A window of 5 % means the peak must have a retention/migration time
between less than 2.5 % and more than 2.5 % of the calibrated
retention/migration time for that peak. For example, a peak with a
retention/migration time of 2.00 in the calibration run must appear between
1.95 and 2.05 minutes in subsequent runs.

For example, an absolute window of 0.20 minutes and a relative window of
10 % gives a retention/migration time window of between 1.80 and 2.20
minutes.

1.80 min = 2.00 min - 0.10 min (0.20 min / 2) - 0.10 min (10 % of 2.00 min).

2.20 min = 200 min + 0.10 min (0.20 min / 2) + 0.10 min (10 % of 2.00 min).

# Corrected Retention/Migration Times

To match peaks by absolute retention/migration times may be simple but not always reliable. Individual retention/migration times may vary slightly due to a small change in conditions or technique. As a result peaks may occur outside the peak matching windows and therefore are not identified.

A technique to deal with the inevitable fluctuations that occur in absolute retention/migration times is to express component retention/migration times relative to one or more reference peaks.

Reference peaks are identified in the calibration table with an entry in the reference column for that peak. The relative peak matching technique uses the reference peak or peaks to modify the location of the peak matching windows in order to compensate for shifts in the retention/migration times of sample peaks.

If no reference peak is defined in the method or the ChemStation cannot identify at least one reference peak during the run, the software will use absolute retention/migration times for identification.

## Single Reference Peaks

A retention/migration time window for the reference peak is created around its retention/migration time. The largest peak falling within this window is identified as the reference peak. The expected retention/migration times of all other peaks in the calibration table are corrected, in proportion to the ratio of the expected retention/migration time to the actual retention/migration time of the reference peak.

## Multiple Reference Peaks

Correcting retention/migration times with a single reference peak is based on the assumption that the deviation of actual retention/migration time from the expected retention/migration times changes uniformly and linearly as the run

progresses. Often during a long run the retention/migration time changes non-uniformly. In such cases better results are obtained using multiple reference peaks spaced at intervals across the run. This splits the signal into separate zones. Within each zone the deviation between retention/migration times is assumed to change linearly, but the rate of change is determined separately for each zone.

**NOTE** The time correction algorithm may fail if the retention times of multiple reference peaks are too close to each other and are not distributed across the total run time.

# Peak Qualifiers

A component can be detected with more than one signal. Although applicable to all forms of chromatography using multiple detectors or detectors capable of producing multiple signals, multisignal detection is most commonly used in liquid chromatography with multiple wavelength or diode array detectors. Such detectors are normally set up so that the wavelength closest to the greatest absorbance (area) is used to define the main peak in the calibration table. In Figure 31 on page 136 this is $Lambda_1$.

The two other wavelengths that were acquired as signals can be used as peak qualifiers. In the figure these are $Lambda_2$ and $Lambda_3$.
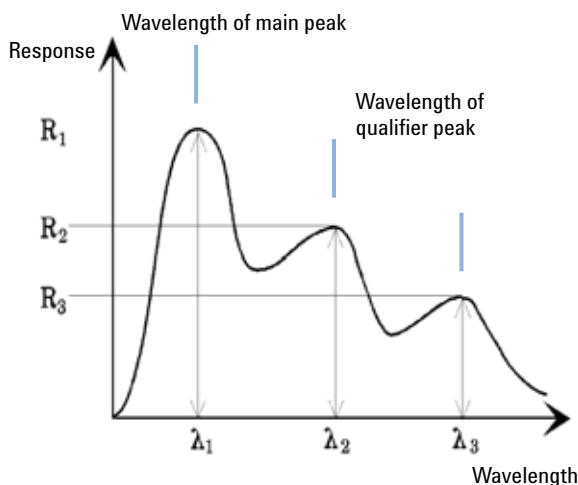


**Figure 31**    Peak Qualifiers

Peaks of a compound have a constant response ratio over different wavelengths.

The qualifier peak response is a certain percentage of the main peak response. Limits which determine the acceptable range for the expected response can be set in the calibration table when the Identification Details option is selected. If the ratio between the main peak qualifier $Lambda_1$ and the qualifier peak, for example, $Lambda_3$ is within the allowed limits then the compound identity can be confirmed.

# Signal Correlation

Signal correlation means that two peaks measured in different detector signals within a defined time window are assigned to the same compound. The signal correlation window can be controlled by the **SignalCorrWin** parameter in the **QuantParm** table of the **_DaMethod** register. Signal correlation is disabled when setting the signal correlation window to 0.0 minutes (see the *Macro Programming Guide* for more information). When signal correlation is off, peaks eluting at the same retention/migration time in different detector signals are treated as different compounds.

The default signal correlation window for LC, CE, CE/MS and LC/MS data is 0.03 minutes and 0.0 minutes for GC data.

# Qualifier Verification

If signal correlation is enabled, qualifier verification is active for all data file types by default. It can be disabled by setting the **UseQualifiers** flag in the **Quantification Parameters** table of the method (see the macro programming guide for more details). Qualifier verification is also disabled when signal correlation is switched off.

# Qualifier Ratio Calculation

When qualifiers verification is enabled for a compound, the ratio of the qualifier size and the main peak size is verified against the calibrated limits. The size may be height or area according to the calculation base setting in Specify Report.

The qualifier peaks can be calibrated in the same way as the target compounds. The user does not need to specify the expected qualifier ratio. The expected qualifier ratio is calculated automatically:

both measured at the retention time of the compound.

The QualTolerance parameter defines the acceptable range of the qualifier ratio, for example, ± 20%.

The tolerance can be set in the calibration table user interface (Identification Details) and is an absolute percentage.

For multilevel calibrations, the ChemStation calculates a minimum qualifier tolerance based on the measured qualifier ratios at each calibration level. The minimum qualifier tolerance is calculated using the following equation:

$$\text{minimum qualifier tolerance} = \frac{\sum_{n=1}^{i}(q_i - \bar{q})}{\bar{q} \times i} \times 100$$

where $q_i$ is the measured qualifier ratio at level i.

# The Identification Process

When attempting to identify peaks, the software makes three passes through the integration data.

## Finding the Reference Peaks

The first pass identifies the time reference peaks. The software searches peak retention/migration times from a run for matches within the retention/migration windows of the reference peaks in the calibration table. A peak from the run is identified as a reference peak in the calibration table if the run peak's retention/migration time is within the window constructed for the calibration table peak.

If more than one peak is found within a window, the peak with the largest area or height followed by a positive signal qualifier match, if set up, is chosen as the reference peak.

After each time reference peak is found, the difference between its retention/migration time and that given in the calibration table is used to adjust the expected retention/migration times of all other peaks in the Calibration table.

## Finding the ISTD Peaks

The second pass identifies any defined internal standard peaks. If they have not already been identified as ISTD, peaks may be identified as time reference peaks. ISTD peaks are identified by peak retention/migration time windows and peak qualifiers. If more than one peak is found in the same ISTD window, the largest peak is chosen.

## Finding the Remaining Calibrated Peaks

The third pass identifies all remaining peaks listed in the calibration table. The non-reference peaks in the calibration table are matched to the remaining run peaks by using their RT window.
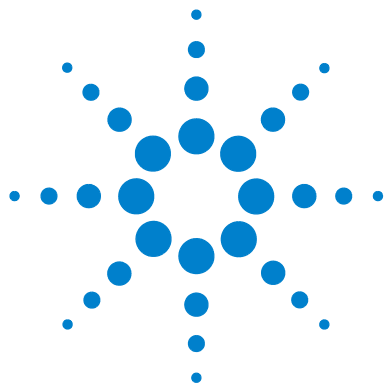
Each non-reference calibrated peak has its own retention/migration time in the calibration table. This is adjusted for the particular run based on the pre-identification of the time reference peaks. The retention/migration time window of the calibrated peak is adjusted based on the corrected retention/migration time of the calibrated peak.

If more than one peak is found in the same window, the peak with a retention/migration time which is closest to the expected retention/migration time and also meets the optional qualifier specifications is chosen.

## Classification of Unidentified Peaks

If there are remaining peaks, which are still not identified, they are classified as unknown. The ChemStation attempts to group the unknown peaks that belong to the same compound. If a peak has been detected in more than one signal, the peaks with the same retention/migration time in each signal are grouped to one compound.

Unknown peaks are reported if the corresponding selection has been made in the Calibration Settings dialog box.

**7**

# Calibration

This chapter describes Calibration in the ChemStation.

**Agilent Technologies**

# Definition of Terms

**Calibration**  Calibration is the process of determining response factors used to calculate absolute component concentrations by injecting specially prepared calibration samples. The calibration table is also used for identification. See "Peak Identification" on page 127.

**Compound**  A chemical compound can comprise several peaks, in a multiple signal calibration, typically one per signal. In a single signal calibration a compound refers to one peak.

**Calibration Level**  A calibration level comprises the calibration points for one calibration sample concentration. In a multisignal calibration the calibration points can be distributed over several signals.

**Calibration Point**  A calibration point refers to an amount/response ratio for a peak on the calibration curve.

**Calibration Sample**  A calibration sample, also referred to as a calibration standard or a standard mixture, is a sample containing a known amount of the compound to quantify. In the software the calibration sample is referred to as an injection from the calibration sample vial.

Calibration samples may be purchased from chemical suppliers or they may be prepared using an accurately measured amount of the pure compound. The amount of the compound in the calibration sample is usually expressed as a concentration, typically in ng/µl units.

# Calibration Table

The calibration table specifies conversions of peak areas or heights into the units you choose according to the calculation procedure you select. It contains a list of retention/migration times from a calibration run. These retention/migration times are compared with retention/migration times of peaks from a sample run. Where a match occurs, the peak in the sample is assumed to represent the same component as that in the calibration table, see "Peak Identification" on page 127. During an analysis or while a report is being generated, the amounts entered for each peak are used to calculate the amounts for the calculation procedure selected for the report. The type and amount of information required for creating a calibration table varies with the type of calculation procedure desired.

The following information is needed to create a calibration table:

- the retention/migration time for each calibration mixture component peak, and

- the amount of each component used in making the calibration mixture, expressed in consistent units.

# Calibration Curve

A calibration curve is a graphical presentation of the amount and response data for one compound obtained from one or more calibration samples.

Normally an aliquot of the calibration sample is injected, a signal is obtained, and the response is determined by calculating the area or height of the peak, similar to Figure 32 on page 144.
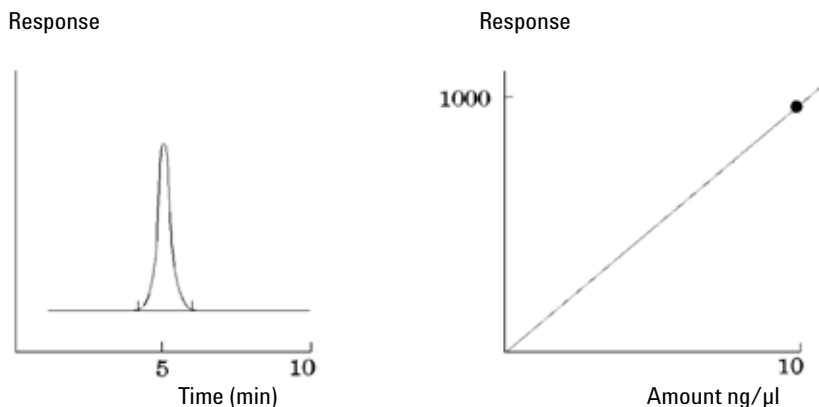
Response                                    Response



**Figure 32**    Calibration Sample (10 ng/µl) Signal and Calibration Curve

A *correlation coefficient* is displayed with the graphic of the calibration curve. The correlation coefficient is the square root of the regression coefficient and gives a measure of the fit of the calibration curve between the data points. The value of the coefficient is given to three decimal places, in the range:

0.000 to 1.000

where:

0.000 = no fit

1.000 = perfect fit

For each calibration level the *relative residual* is displayed. It is calculated using the following formula:

$$relRES = \frac{Response_{calibrated} - Response_{calculated}}{Response_{calculated}} \cdot 100$$

where:

relRES = relative residual in percent

The calculated response represents the point on the calibration curve.

The *residual standard deviation,* which is printed on some reports and when selecting Print calibration table and curves is calculated using the following formula:

$$ResSTD = \sqrt{\frac{\sum\limits_{i=1}^{n} (Resp_{calibratedi} - Resp_{calculatedi})^2}{n-2}}$$

where:

ResSTD = residual standard deviation

Respcalibratedi = calibrated response for point i

Respcalculatedi = calculated response for point i

n = number of calibration points

# Unknown Samples

An unknown sample is a sample containing an unknown amount of the compound to be quantified.

To find out how much of the compound is in the unknown sample, you must:

- create a calibration curve for the compound,

- inject an aliquot of your unknown sample and run the analysis in exactly the same way as for the calibration sample,

- determine from the signal the response, which is the area or height of the peak due to the unknown amount of the compound, and

- use the calibration curve to calculate the amount of the compound in the unknown sample.

For example, if the area of peak in the unknown sample is 500, you can determine that the amount in the unknown is 5 ng/µl, by using the calibration curve shown in Figure 33 on page 146.
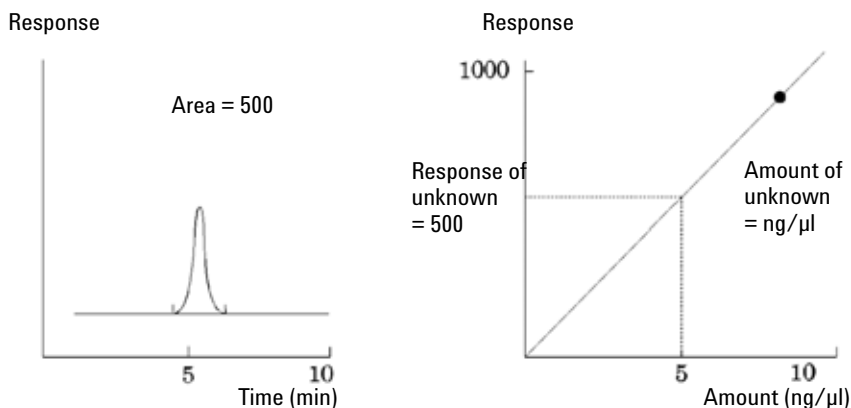


**Figure 33** Signal from Unknown Sample and Calibration Curve

# Types of Calibration

The ChemStation offers two types of calibrations, single-level and multilevel calibrations.

## Single-Level Calibration

The calibration curve shown in Figure 34 on page 147 contains one point, that is, one level. For the single-level calibration curve, the response of the detector is assumed to be linear over the working range of concentrations for the samples of interest. The response factor for a given component peak is given by the inverse of the slope of the calibration curve line through the point and the origin. A disadvantage of single-level calibration is that the detector response to the sample concentration is assumed to be linear and pass through the origin on a concentration versus response plot. This is not always true and can lead to inaccurate results.
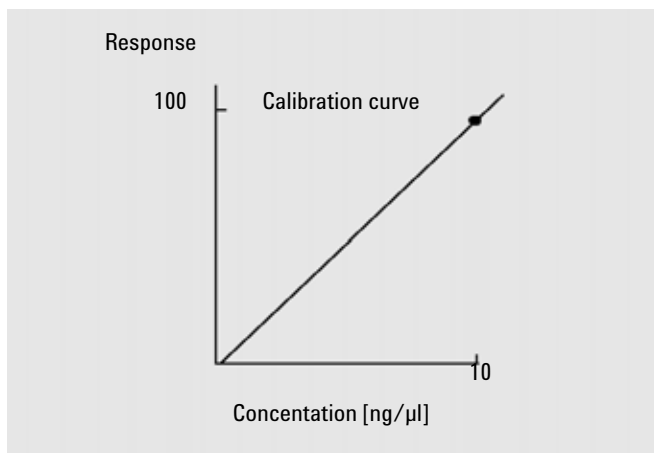


**Figure 34**     Single-level calibration curve

To obtain accurate quantitative results, a calibration curve should have at least two levels. These levels should bracket the amounts expected to be found in the unknown samples.
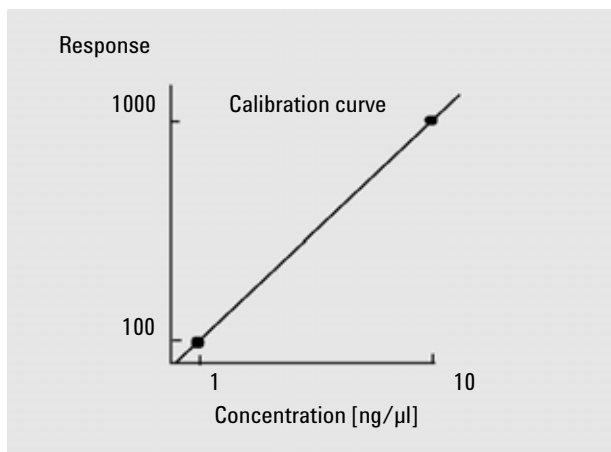
**Figure 35**     Two-level calibration curve

For example, if you want to quantify a compound, and the unknown samples are expected to range from 1 – 10 ng/µl, then a calibration curve should have at least the two levels as shown in Figure 35 on page 148.

### Amount Limits

The ChemStation allows you to define the valid quantification ranges in terms of absolute amounts for each component.

## Multilevel Calibration

Multilevel calibration can be used when it is not sufficiently accurate to assume that a component shows a linear response or to confirm linearity of the calibration range. Each calibration level corresponds to a calibration sample with a particular concentration of components. Calibration samples should be prepared so that the concentration of each component varies across the range of concentrations expected in the unknown samples. In this way it is possible to allow for a change in detector response with concentration and calculate response factors accordingly.

This multilevel calibration curve has three levels and shows a linear fit through the origin. This method of linear fit through the origin is similar to the single-point method calibration. The detector response to concentration is

assumed to be linear. The difference between the two calibration types is that, with linear fit, the slope of the detector response can be determined by a best fit through a number of points, one for each level.
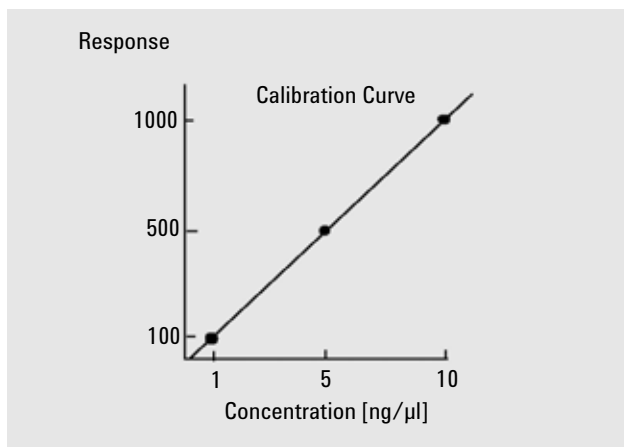


**Figure 36**     Multi-level calibration curve with three levels

The corresponding calibration table, which is the tabulation of the information used to generate this curve, might look similar to the one shown in

**Table 13**     Calibration Table

| Level | Amount (ng/µl) | Response (area counts) |
|-------|----------------|------------------------|
| 1     | 1              | 100                    |
| 2     | 5              | 500                    |
| 3     | 10             | 1000                   |

In this example, the calibration samples that were used to generate the three levels had been identified as 1, 2 and 3.

# Calibration Ranges

Each multilevel calibration is valid over the range of concentrations used in the calibration samples. Extrapolation of a calibration curve, especially if it is non-linear, is at best an approximation. The valid calibration range for each compound may be defined in the Compound Details dialog box. Each entry for that compound can be expressed as lower and upper limits. If these limits are exceeded, the report is annotated.

# Calibration Curve Fits

Various curve-fit calculations are available for use with multilevel calibration.

- Piecewise Linear
- Linear
- Log
- Power
- Exponent
- Quadratic
- Cubic
- Average (Response/Amount)

### Non-Linear Fit

In some cases, the detector response to changes in sample concentration is not linear. For these types of analyses, a linear regression calibration method is not appropriate and a multilevel calibration calculation should be used.

# Origin Treatment

There are four ways to treat the origin when the response curve is plotted:

- ignore the origin,
- include the origin,
- force the origin, or
- connect the origin.

To force the origin to be included in the calibration curve the calibration points are mirrored about the origin from the first quadrant into the third quadrant. Using all points for the regression calculation ensures that the resulting calibration curve passes through the origin. This is also explained in Figure 37 on page 151.
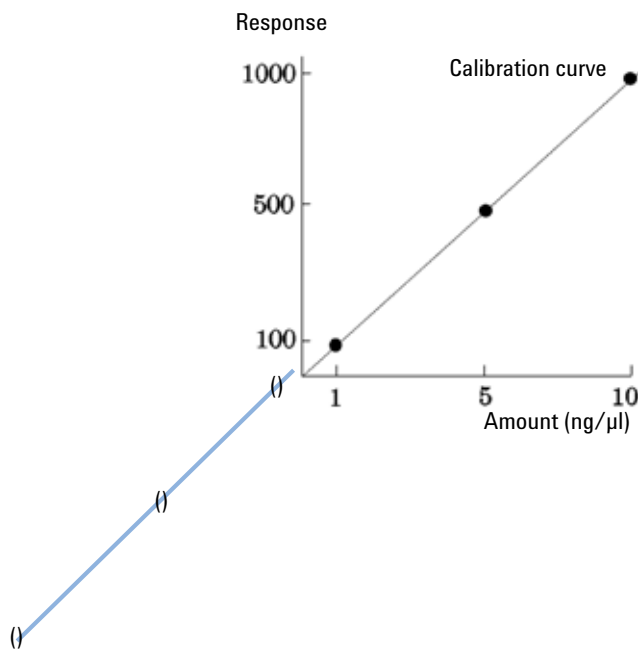


**Figure 37**   Forcing the origin to be included

For further information about calibration curve fits and origin treatment, see the *online help* file.

## Calibration Point Weighting

When setting up your default calibration curve, you can specify the relative weighting (or importance) of the various calibration points used to generate the curve.

The following weighting options can be selected:

| Weight | Description |
|---|---|
| Equal | All calibration points have equal weight in the curve. |
| Linear (Amnt) | A calibration point with the amount x has the weighting $1/x$ normalized to the smallest amount so that the largest weight factor is 1. Normalization is done by multiplying the weight with the smallest amount. For example the weight of a calibration point with the amount x is $(1/x) \times a$ where a is the smallest amount of the calibrated compound prepared in the calibration standards. <br> If the origin is included it is assigned the mean of the weightings of the other calibration points. |
| Linear (Resp) | A calibration point with the response y has the weighting $1/y$ normalized to the smallest response so that the largest weight factor is 1. Normalization is done by multiplying the weight with the smallest response. For example the weight of a calibration point with the amount y is $(1/y) \times b$ where b is the response corresponding to the smallest amount of the calibrated compound prepared in the calibration standards. <br> If the origin is included it is assigned the mean of the weightings of the other calibration points. |
| Quadratic (Amnt) | A calibration point with the amount x has the weighting $1/x^2$ normalized to the smallest amount so that the largest weight factor is 1. Normalization is done by multiplying the weight with the smallest amount. For example the weight of a calibration point with the amount x is $(1/x^2) \times a^2$ where a is the smallest amount of the calibrated compound prepared in the calibration standards. |
| Quadratic (Resp) | A calibration point with the response y has the weighting $1/y^2$ normalized to the smallest response so that the largest weight factor is 1. Normalization is done by multiplying the weight with the smallest response. For example the weight of a calibration point with the response y is $(1/y^2) \times b^2$ where b is the response corresponding to the smallest amount of the calibrated compound prepared in the calibration standards. |
| # Calibrations | A calibration point is weighted according to the number of recalibrations of the point. No normalization is done. |

Quadratic calibration point weightings, for example, can be used to adjust for a spread in calibration points. It makes sure that calibration points closer to the origin, which can normally be measured more accurately, get a higher weight than calibration points further away from the origin, which may be spread.

You should base your decision, which kind of calibration point weighting to use, on your method requirements.

# Group Calibration

Group calibration can be applied for compounds where the individual concentrations are not known but the sum of concentrations for a group of compounds is known. An example are isomers. Complete compound groups are calibrated. The following formulae are used:

Calibration

$$Conc_{AB} = RF_A \cdot Response_A + RF_B \cdot Response_B$$

where:

$Conc_{AB}$ is the concentration of the compound group consisting of compound A and B

$Response_A$ is the area (or height) of compound A

$RF_A$ is the response factor

For compounds within a compound group we assume equal response factors:

$$RF_A = RF_B$$

Therefore the concentration of a compound within a compound group is calculated as follows:

$$Conc_A = \frac{Conc_{AB} \cdot Resp_A}{Resp_A + Resp_B}$$

# Peak Summing

The peak sum table is provided for certain applications in the petrochemical and pharmaceutical industries that can be performed more effectively with the following features:

• Sum the areas of peaks that lie within a range specified by the user

• Sum the areas of a range of peaks and perform the calculations using a single multiplier

• Sum the areas of all peaks having the same name

The peak sum table is similar to, but distinct from the standard calibration table. Like the calibration table, it is associated with the current method.

| | |
|---|---|
| **N O T E** | You must create a calibration table for an analysis before you can create the peak sum table. |

# Recalibration

## What is Recalibration?

Recalibration is the process used when you want to update a level on a calibration curve. When you recalibrate you run another sample that contains the same calibration compounds as the original, and most important, the same amount of these compounds. When you run the calibration sample, you obtain updated response factors and retention/migration times. You may also choose to average the response factors over a number of calibration runs so that response factors are weighted equally.

## Why Recalibrate?

Most calibrations have a limited lifetime, due to changes in chromatography. Recalibration is necessary to maintain the accuracy of the analysis. For example, assume you have created a calibration table for the compound caffeine which you use whenever you are required to quantify samples containing caffeine. At some point you will need to replace the column/capillary. Although the column/capillary is replaced with exactly the same type, it will not behave in exactly the same way as the previous column/capillary when you first created the calibration table for caffeine. Therefore, to ensure consistency you should recalibrate the levels in the calibration table before using the new column/capillary to analyze samples containing unknown amounts of caffeine. By doing this you are quantifying samples analyzed under the same system conditions.

## Manual Recalibration

You can enter peak calibration information manually and normalize the calibration table using the Manual Setup option button in the New Calibration Table dialog box. Typically, a new calibration method is produced by running a calibration standard mixture, creating a calibration table, and entering the amounts of all calibrated peaks to obtain response factors. This approach is inefficient for some application, such as found in the petrochemical industry,

where the same compounds have been analyzed for many years and the response factors for various compounds and detectors are readily available.

You create the calibration table manually by entering peaks and their response factors into the calibration table, recalibrating the method using a standard that contains at least one response reference peak, and selecting Delta% update.

# Recalibrations with Peak Summing

When a recalibration is performed, the retention/migration time ranges in the Peaksum Table of the method will be updated before the actual recalibration is done. Peak Sum recalibrations are performed in this way to ensure the delta is incorporated into the time calculations.

# Recalibration Options

You have several ways to update the responses in the calibration table with the new calibration data.

### Average

The average from all calibration runs are calculated using the following formula

$$Response = \frac{n \cdot Response + MeasResponse}{n + 1}$$

### Floating Average

A weighted average for all calibration runs is calculated. The updated weight is set in the Recalibration Settings dialog box.

$$Response = \left(1 - \frac{Weight}{100}\right) \cdot Response + \left(\frac{Weight}{100}\right) \cdot MeasRespons$$

### Replace

The new response values replace the old values.

# Ways to Recalibrate

Recalibration can be done in two ways using the ChemStation software. You can recalibrate interactively or automatically during a sequence of automated analyses. Interactive recalibration is when you directly go through the process of recalibration using the ChemStation software after injecting one or more calibration samples. Recalibration using a sequence is where you specify when the recalibration occurs, but the automation software does the recalibration. For more information refer to "Automatic Recalibration" on page 179.

For information on how to perform recalibration using the software, see the How To part of the help system.

# Recalibration of Unidentified Peaks

There are three ways to recalibrate unidentified peaks.

### No Recalibration

If a peak in the calibration table cannot be identified in the integration results, the calibration is aborted. If this occurs in a sequence, the sequence is also aborted.

### Partial Recalibration

This feature allows the recalibration of only the identified peaks. If peaks are missing, the calibration is not aborted but the report is annotated that peaks are missing.

### Recalibration of all Retention/Migration Times

This feature allows the retention/migration time recalibration of all identified and unidentified peaks. This is done by using the retention/migration times of the identified peaks. There is no update of response factors for unidentified peaks.

# 8

# Automation

**Agilent Technologies**

This chapter describes the concepts of automation. It explains how to work
with sequences in ChemStation, what happens when a sequence is run and
how to customize sequences.

# What is Automation?

Automation is the unattended analysis of more than one injection.

The sequence part of the ChemStation software allows you to automate acquisition, data evaluation, and report generation.

# What is a Sequence/Sequence Template?

A sequence is a series of instructions that automates the analysis of samples.

A sequence can be used to automatically inject each sample, and to acquire and analyze the data according to the method specified for that sample. Each sample vial in a sequence may be analyzed with a different analytical method and, thus, use different sets of chromatographic/electropherographic conditions and evaluation parameters.

ChemStation introduces two data storage modes, to allow you to choose the data storage model that matches your workflow. These modes have influence of the sequence usage:

- Unique Folder Creation ON
- Unique Folder Creation OFF

Unique Folder Creation ON for consistency of sample data uses sequences as "sequence templates" that can be used to run the acquisition multiple times; however, they are not used for reprocessing in Method and Run Control. When the sequence template is run, a sequence data container is created, containing all related files. If the sequence template is reused, a new sequence data container is created for each reuse

Unique Folder Creation OFF stores all data in one directory. The sequence *.s files are not used as sequence templates, so re-executing a sequence again might overwrite present data, if the data directory is not changed from the user.

The available sequence/sequence templates (*.s) are visible in the ChemStation Explorer. For quick and easy navigation, you can add additional sequence/sequence template locations to the ChemStation Explorer selection tree using the Paths tab of the Preferences dialog box.

# Preferences - Sequence Tab

On the Sequence tab, the user has the choice of two different data storage models. These modes define how the sequence data are stored in the ChemStation.

## Unique Folder Creation ON

In this mode of data storage, there is a robust and permanent link between the raw data and the method. Each data file, whether acquired within a sequence or as a single run, has links to at least two methods: the method that was used to acquire the data, and the one that was used for data analysis.

The sequence data are stored in a sequence data container using a defined sequence container name. You can specify the naming conventions (Name Pattern) for these sequence containers in the Sequence tab of the Preferences dialog box. If no name pattern is specified, a default sequence name pattern is used. The Sequence Tab is used only for data acquisition and therefore is present only for online systems.

The sequence name pattern can contain various sections. The system creates a name for your sequence data container that is determined by the sequence name pattern sections you select. All data files, methods, the sequence logbook, the sequence_name.s file and the sequence_name.b file belonging to this particular sequence are stored in the sequence data container. The sequence data container is created when the sequence is started.

The sequence (.s) files are used as sequence templates, and this concept allows you to run any sequences.s file multiple times without overwriting existing data and without changing the sequence parameters. If neither counter nor time are used in the sequence name pattern, the system introduces a counter automatically to avoid overwriting data. For the second, third and all subsequent sequences using the same sequence template, a counter is added to the sequence container name.

## Unique Folder Creation OFF

In this mode of data storage, the method name is the only link that exists between the data file and the method that was used to acquire and process it. No copies of the method are saved with the sequence or with the data file; if the method is changed, or a new method with that name is created, then the sequence cannot be exactly reproduced.The sequence data files are stored in accordance with the parameters specified in the Data File group of the Sequence Parameters dialog box; the sequence naming facility in the Sequence tab of the Preferences dialog box is disabled in this mode.This mode of data storage is identical to ChemStation revisions prior to B.02.01 and therefore cannot take full advantage of the latest Data reviewing/reprocessing functionalities in the Data Analysis View of the ChemStation.

**NOTE** Sequence data acquired using the option "Unique folder Creation OFF" need to be reprocessed using the reprocess option in the Method and Run Control view.

**NOTE** The Add-On solutions G2189BA ChemStation OpenLAB Option and G2181BA ChemStore require the preference mode "Unique Folder Creation ON". As soon as the Add-On is installed on top of the ChemStation, the option "Unique Folder Creation OFF" is disabled.

# Sequence Parameters

The **Sequence Parameters** dialog box contains information common to all sample vials in a sequence. Use this dialog box to:

- select the data directory using the **Path** combo box, and enter information about the operator name (the operator name entered in the **access level** dialog box is shown), and
- specify how the sequence processing should be done by choosing particular part of Methods and Run parameters.

For example, you can choose to either:

- execute the run-time checklist,
- do acquisition only, or
- do reprocessing only - for data acquired till ChemStation Rev. B.01.03. - for data acquired with option **Unique Folder Creation OFF**

| NOTE | Sequence data acquired with ChemStation revisions up to B.01.03 or acquired using the options **Unique folder Creation OFF** need to be reprocessed using the **reprocess** option in the **Method and Run Control** view. |

Sequence data acquired with ChemStation revisions B.02.01 and higher need to be reprocessed using the **reprocess** option in the **Data Analysis Navigation table**.

If the **reprocess** option is selected, you have the choice to use the sample data defined when the sample was originally analyzed, or by activating the **Use Sequence Table information** check box, you can use updated sample data by entering new data in the sequence table:

- specify what happens when the sequence is finished using the **shutdown** parameters, and
- specify if the barcodes should be used in the sequence and how to handle a barcode mismatch, assuming that a barcode reader is installed on the system.

# Sequence Table

The sequence table determines which methods are used to analyze the sample vials and the order in which the vials are analyzed. This table also contains information about each sample, including the name, quantification parameters, and recalibration parameters.

The injector group box is displayed for instruments that support dual sampling (GC). Selecting Front or Back displays the lines in the sequence table and the currently running status for that injector.

For a description of the columns in this table and how they interact with information stored with the method, see the online help reference.

# Creating Sequences (Sequences and Sequence Templates)

Use the sequence table to specify the samples, methods, and vials to run in the sequence. The sequence table lists each sample in the sequence in the order it will be run and contains the necessary vial, method, and calibration information for each sample.

## Using the Sequence Table Editor

If you would like to change the view and content of your sequence table, you can open the Sequence Table Editor by clicking the listing symbol in the lower right corner of the Sequence Table. The Sequence Table Editor opens and allows you to specify if a certain column is shown within the Sequence table. In addition the column widths can be defined for each sequence table column. Depending on the installed software packages, additional column fields will be added, for example, "Target Mass" field if an LC/MS is installed, "Study" field for ChemStore Add-On installation.

## Using the Insert Vial Range Button

If you have many samples that use the same method, you can quickly enter these samples into the Sequence Table using the Insert Vial Range feature. This feature copies the method name, vial range, number of injections per vial, and if you specify the Sample Amount, ISTD Amount, Multiplier, and Dilution. The system then enters the information for each vial in the range into the sequence table.

## Using the Append Line Button

To append a new blank line to the end of the sequence table, select the Append Line button.

## Using the Custom Fields Button

If custom fields have been set up in the method(s) used in the sequence table, select the Custom Fields button in order to edit the custom fields values for each sample (sample related custom fields) or for each compound in the method of a sample (compound related custom fields).

# Working with Sequences (Sequences and Sequence Templates)

Sequences (Sequences and Sequence Templates) are accessed and created from the Sequence menu. Sequences may be created and saved in the same way as methods. When you save a sequence, a file is created with a .S extension. When you want to edit or use the sequence again, you access it by, for example, using the Load Sequence item in the Sequence menu.

## Priority Samples

A currently running sequence can be paused after the current method is completed. The sequence can be paused to allow the analysis of a priority sample by the same or another method. The sequence can then be resumed, where the sequence continues with the sample at which the sequence was paused.

## Sequencing with Control Samples

Control samples can be specified in the Sample Type field of the sequence table. The method used to analyze the control sample must contain a calibration table where control sample limits for one of the compounds are specified. If the control sample limits you specified are exceeded, the sequence is stopped and a message is written into the logbook. If you are using one of the ChemStation report styles, the control sample limits are also printed on the reports generated for these analyses. For more information on how to define a sequence with control samples, see the How To section of the online help system.

## Stopping a Sequence

The currently active run will be completed before the sequence stops. A stopped sequence can never be resumed.

## Aborting a Sequence

The abort function terminates a currently active sequence immediately.

## Pausing a Sequence

During a sequence pause, the sequence table file name and the data file name cannot be changed. In the sequence table you can only change sequence lines that have not already been executed or change the vial number in the current sequence line. You can add, delete, and change sequence lines for future analyses.

For example, it may be necessary to edit an active sequence to add a new batch of samples. You can edit the sequence so these vials will be the next sample that the ChemStation will process after the samples in the currently running sequence line.

## Running a Partial Sequence

A sequence table that is already set up can be partially executed by selecting Partial Sequence from the Sequence menu. The system displays the Partial Sequence dialog box and allows you to select individual samples from the table for analysis.

A single run is shown on each line of the Partial Sequence dialog box. For each run, the vial, method, data file, and sample name are given. Additionally, encoded information about the sequence table and any calibration samples are shown in the Seq Tbl and Calib:RF:RT columns. See the online help for an explanation of these codes

You can obtain a paper copy of the partial sequence by selecting the Print button.

The following Partial Sequence dialog box results when the SimpReg Method and Sequence Table, shown later in Table 19 on page 190 and Table 20 on page 191, are current. Samples 1, 2, 4, 5, and 8 are marked for processing.

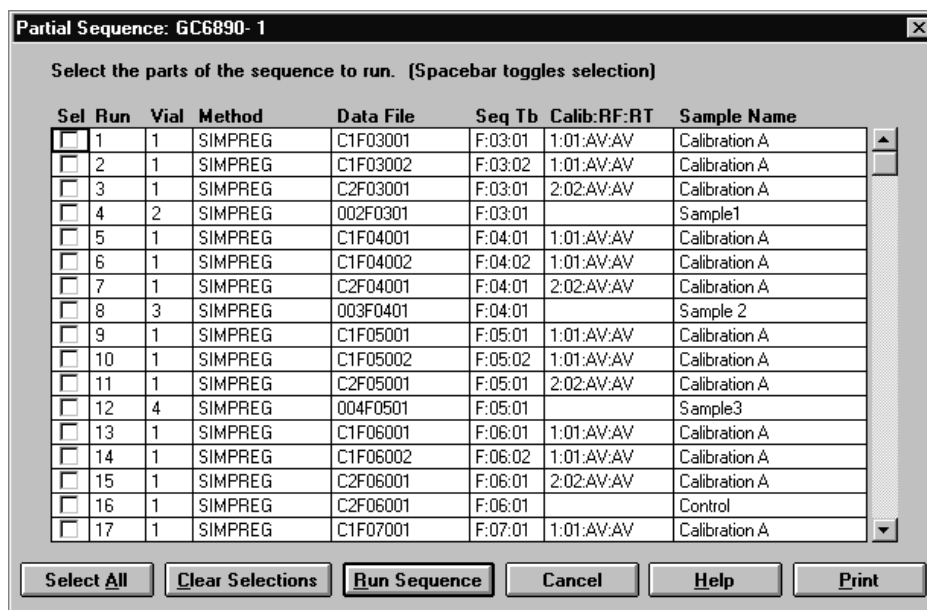**Figure 38**   Partial Sequence Dialog Box

### Partial Sequence in Preference Mode "Unique Folder Creation ON"

The sequence data are stored in a sequence data container using a defined
sequence container name. If you run a partial sequence the system will create
a new sequence data container based on the Preference settings, each time a
part of that sequence is executed. So it is possible to create e.g. three sequence
data container of the same sequence by executing three time a partial
sequence based on the one sequence.

### Partial Sequence in Preference Mode "Unique Folder Creation OFF"

The sequence data files are stored in accordance with the parameters
specified in the Data File group of the Sequence Parameters dialog box. Even
if the sequence is executed partially, all data files will be stored in the same
subdirectory.

# Sequence Log File

A sequence log file is produced that indicates what has happened during the running of the sequence. It is useful for identifying when errors occurred if the sequence is running unattended or overnight. The name of the logbook file always has the .log extension. The logbook file is located in the directory where the data of the sequence is stored.

# What Happens When a Sequence is Run?

### Starting a Sequence using "Unique Folder Creation ON"

The system creates a sequence data container based on the path definition in the sequence parameters and the sequence preference settings. The sequence template *.s, all methods defined in the sequence table belonging to this particular sequence are copied to the sequence data container. The system continue to work with these files during acquisition. Starting the sequence, the method of the corresponding sequence line is loaded within ChemStation from out these data container.

### Starting a Sequence using "Unique Folder Creation OFF"

Starting a sequence, the system is loading the sequence *.s file and based, on the entry in the sequence table the corresponding method of the sequence line is loaded within ChemStation. In contract to the second data storage mode "Unique Folder Creation ON", no sequence data container is created. Sequence and methods will remain in their master directory.

### Further executed steps during sequence execution:

The following steps will be repeated for each executed sequence line:

- If equipped with an autosampler, the ChemStation software first locates the sample in the autosampler according to the number entered in the vial column
- The instrument is loaded with the method parameters.
- The pre-run macro is executed.
- The sample is then injected into the instrument (manually or automatically).
- The method data evaluation is done. Integration, quantification, and reporting, including any user specified macro. In case the mode "Unique Folder Creation ON" is used, the system is storing two additional methods, ACQ.M and DA.M during the run.
- The post-run macro is executed.

- During the entire process, the ChemStation tracks the sequence's progress in real time and produces a sequence log file.

**ChemStation status**
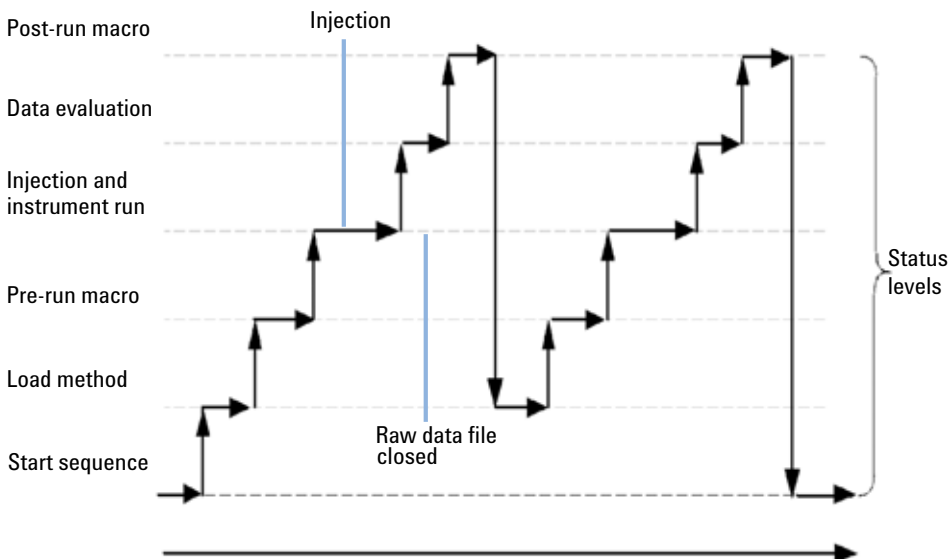


**Figure 39** Sequence Status

# Sequence Data File Structure (Unique Folder Creation ON)

In the ChemStation revision B.02.01 and later, the link between the raw data and the method has been strengthened, as shown in Figure Sequence Data File Structure "Unique Folder Creation ON".
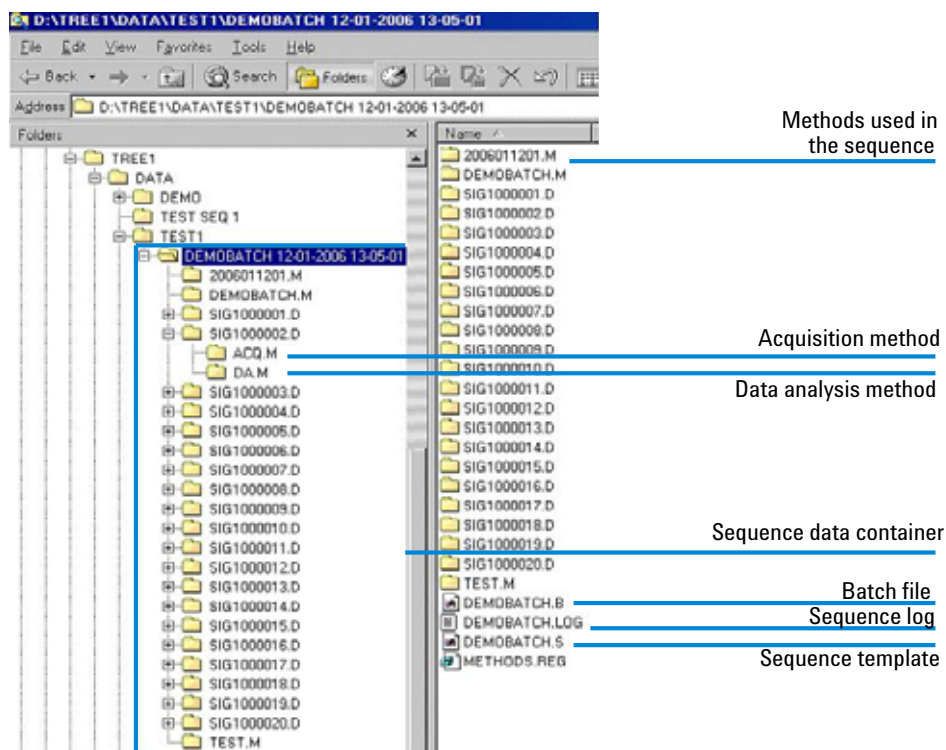


**Figure 40**    Sequence Data File Structure

# Data File Naming in a Sequence

Data file naming in a sequence can be done in the following ways:

- automatic,
- manual, or
- prefix/counter.

## Automatic Data File Naming During a Sequence

### Sample Vials

For example 017-0103.D

where:

- The first three digits are the vial number, for example, 017.
- The fourth digit in liquid chromatography and capillary electrophoresis is a separation hyphen (-); in a gas chromatograph, this will be either (F) for front or (B) for back.
- The fifth and sixth digits are the sequence line that defines the method used, for example, 01 for the first sequence line.
- The seventh and eighth digits are the injection number for this vial by the method, for example, 03 for the third injection.

### Blank Runs

For example NV--0499.D

where:

- NV stands for no vial.
- - is a separation hyphen.
- 0499 is the 99th blank run of sequence line 4.

# Entering Data File Names Manually

One of the columns in the sequence table is named Datafile. When it contains no entry, the data file naming scheme specified (automatic or prefix-counter) is used to create the data file name. If any text is entered into the Datafile column, the ChemStation uses that text as the data file name for the run.

If more than one injection per vial is specified on a line with a manual data file name, the ChemStation automatically truncates characters from the end of the name entered by the user and appends the injection number. This prevents the same data file name from being reused for multiple injections.

## Using a Prefix/Counter to Name Data Files

If you use the Prefix/Counter to name data files, the ChemStation generates a name for each analysis. For an instrument that supports dual signal analysis such as the GC, the ChemStation will generate a name for each signal.

The sequence set up allows long filenames for the prefix/counter. The datafile name defined by prefix/counter can have up to fifteen characters plus .d extension, so in total seventeen characters.

Following rules apply to the prefix/counter field:

• the counter itself can have a maximum up to 6 characters

• if a prefix provides less than nine characters for prefix, the counter is automatically extended to 6 digits

• the number given in the counter is the start number being incremented

**Table 14**

| Prefix | Counter | results in file name |
|---|---|---|
| long | 000001 | long000001 |
| longname | 000001 | longname000001 |
| testwithalongna | 1 | testwithalongna1 |

# Postsequence Operation

You can specify what happens after a sequence has finished during normal execution or when the ChemStation encounters an error during sequence operation. For LC operation, this is done by activating the post-sequence Cmd/Macro check box of the Sequence Parameters where you have a choice of:

- Setting the system to a STANDBY state where the pump and lamp are off,
- setting the system to a LAMPOFF state where all lamps are off (LC and CE only),
- Setting the system to a PUMPOFF state where all pumps are off (LC and CE only), or
- Using a default SHUTDOWN macro or modifying SHUTDOWN.MAC to determine specific operation.

  For example, you may want to switch off your system after your sequence has completed. Other uses of the shutdown macro could be to set your flow to zero or slowly reduce your flow.

In the Sequence Parameters, you may specify any custom macro to run by including its name in the Post-Sequence Cmd/Macro field and checking the box.

## Not Ready Timeout (LC and CE only)

The Not Ready Timeout in the Sequence Parameters is the length of time the system will wait for the instrument to become ready — after this time the system will shut down.

## Wait Time (LC and CE only)

Sequence Parameters allows you to specify a wait time which is executed after loading method and before injecting with that method. This can be useful for column/capillary re-equilibration when using new analysis conditions.

# Automatic Recalibration

Calibration is often done after a change in operating conditions, for example, after changing a column or capillary. Automatic recalibration is usually done at the start of a sequence of analyses or at regular intervals during a sequence as part of a program to compensate for factors that affect the analytical performance.

There are two ways to specify automatic sequence recalibration:

- explicit calibration sequences, or
- cyclic calibration sequences.

### Recalibration using Preference Mode "Unique Folder Creation ON"

While performing a recalibration, the calibration table of the used method is update according the defined method settings. Using the data storage mode "Unique Folder Creation ON", the recalibrated methods are available within the sequence data container. The calibration table of the sequence method is updated during that process. In addition the DA.M method of the individual data files contains the updated calibration used for result creation.

### Recalibration using Preference Mode "Unique Folder Creation OFF"

While performing a recalibration, the calibration table of the used method is update according the defined method settings. Using the data storage mode "Unique Folder Creation OFF", the calibration table of the master method is update during the recalibration.

# Specifying Recalibrations

The recalibration parameters for the sequence are entered directly into the sequence table. These parameters define how the method is recalibrated during a sequence.

## Recalibration Parameters in the Sequence Table

The response factor and retention/migration times can be updated in several ways. The calibration level, update response factor and update retention/migration times are instructions used in the data analysis when recalibrating the calibration table.

When Calibration is entered in the SampleType column of the sample table, the following columns become active and can be edited:

• Cal Level

• Update RT

• Update RF

• Interval

The values that can be entered into each of these columns are shown in the table.

**Table 15** Recalibration Parameters in Sequence Table

| CAL Level | Update RT | Update RF | Interval |
|---|---|---|---|
| Calibration table Level # (1-999) | No update | No update | Cyclic recalibration interval # (1-999) |
| | Average | Average | Blank |
| | Replace | Replace | |
| | | Bracket | |
| | | Delta% | |

The table shows the columns in the sequence table that contain the recalibration parameters and the values that can be entered.

## No Update

Does not change the response factor or retention/migration time.

## Replace

Replaces the previous retention/migration times and the response (areas or heights) with those from the current run only. The response is not changed for any peak that is not found in this recalibration run.

## Average

Averages the retention/migration times and responses (areas or heights) for each peak based on the original calibration run and all averaged recalibrations since then. If a peak is missing in one of the recalibrations, the average response of the peak will not suffer.

## Bracket

The samples are bracketed by pre-sample and post-sample calibrations. Evaluation is done when the last calibration sample of the closing bracket has been run. The existing calibration data is replaced by the result data of the calibration run of the opening bracket. The closing bracket calibrations are averaged to that calibration table.

## Interval

The Interval determines how often a calibration is done during a sequence. Calibration frequency corresponds to the number of sample injections that are done before the next set of calibration injections takes place. At the start of the analysis, a calibration is done and the results (response factors) are entered into the calibration table. These results are then used in subsequent quantitative calculations. After the specified number of injections have been done, another calibration is made and the results are entered into the calibration table overwriting the results of the previous calibration run.

## Delta%

The delta% calculation allows you to compare response factors from an analysis with response factors entered manually into a calibration table. The delta% is then applied to all of the calibrated peaks in the table. You can identify several internal standards, and their measured response factors are then used to calculate new response factors for the other peaks. You identify which internal standard is to be used for the delta% calculation for each peak in the calibration table.

# Types of Sequences

The following are types of sequences:

- explicit calibration sequences,
- explicit single-level calibration sequences,
- cyclic multiple-level calibration sequences,
- explicit and cyclic calibrations together in a sequence, and
- cyclic calibration sequences with bracketed calibrations.

# Explicit Calibration Sequences

This type of sequence recalibrates at defined intervals specified by you in the sequence table.

For explicit calibration sequences, the calibration samples are entered in the sequence without an interval entry in the sequence table. A recalibration is done once for each calibration sample entry in the sequence table.

# Cyclic Single-Level Calibration Sequences

This type of sequence uses the same vial, that is, the calibration sample at regular intervals in the sequence.

The interval entry in the sequence table determines how the recalibration is done. For example, an interval value of 2 will recalibrate after every two sample vials in the sequence.

# Cyclic Multiple-Level Calibration Sequences

This type of sequence uses different calibration samples to recalibrate a multiple-level calibrated method.

The following example describes a two-method sequence comprising method A and method B to analyze two groups of samples. Both methods are multiple-level calibration methods that will recalibrate automatically at defined intervals.

For each method the Sequence Table has three entries:

- Two calibration levels:
  - Sequence lines 1 and 2 in method A.
  - Sequence lines 8 and 9 in method B.
- Five entries for the samples:
  - Sequence line 3 through 7 in method A.
  - Sequence line 10 through 14 for method B.

The calibrations are specified at regular intervals by the recalibration interval entry in the Sequence Recalibration Table.

- Method A will recalibrate after every two samples.
- Method B will recalibrate after every three samples.

The Sequence Table below is truncated to simplify the example.

**Table 16**    Sequence Table for Method A and Method B

| Line | Vial | Method Name | Inj/Vial | Sample Type | Cal Level | Update RF | Update RT | Interval |
|------|------|-------------|----------|-------------|-----------|-----------|-----------|----------|
| 1 | 1 | Method A | 1 | Calibration | 1 | Average | No update | 2 |
| 2 | 2 | Method A | 1 | Calibration | 2 | Average | No update | 2 |
| 3 | 10 | Method A | 1 | | | | | |
| 4 | 11 | Method A | 1 | | | | | |
| 5 | 12 | Method A | 1 | | | | | |

**Table 16**    Sequence Table for Method A and Method B

| Line | Vial | Method Name | Inj/Vial | Sample Type | Cal Level | Update RF | Update RT | Interval |
|------|------|-------------|----------|-------------|-----------|-----------|-----------|----------|
| 6 | 13 | Method A | 1 | | | | | |
| 7 | 14 | Method A | 1 | | | | | |
| 8 | 3 | Method B | 1 | Calibration | 1 | Average | No update | 3 |
| 9 | 5 | Method B | 2 | Calibration | 2 | Average | No update | 3 |
| 10 | 20 | Method B | 1 | | | | | |
| 11 | 21 | Method B | 1 | | | | | |
| 12 | 22 | Method B | 1 | | | | | |
| 13 | 23 | Method B | 1 | | | | | |
| 14 | 24 | Method B | 1 | | | | | |

# Method A Analysis Order

This section describes the analysis order for Method A which is the first part of the two-method sequence.

**Table 17**    Method A Analysis Order

| Inj No. | Method | Vial | Operation |
|---------|--------|------|-----------|
| 1 | Method A | 1 | Calibration level 1 and report |
| 2 | Method A | 2 | Calibration level 2 and report |
| 3 | Method A | 10 | Sample analysis and report |
| 4 | Method A | 11 | Sample analysis and report |
| 5 | Method A | 1 | Calibration level 1 and report |
| 6 | Method A | 2 | Calibration level 2 and report |
| 7 | Method A | 12 | Sample analysis and report |
| 8 | Method A | 13 | Sample analysis and report |

**Table 17** Method A Analysis Order

| | | | |
|---|---|---|---|
| 9 | Method A | 1 | Calibration level 1 and report |
| 10 | Method A | 2 | Calibration level 2 and report |
| 11 | Method A | 14 | Sample analysis and report |

# Method B Analysis Order

This section describes the analysis order for Method B, which is the second part of the two-method sequence.

Method B has the following differences compared with Method A:

- There are two injections per vial for calibration level 2. The Interval entry is set to 3.

**Table 18** Method B Analysis Order

| Inj No. | Method | Vial | Operation |
|---|---|---|---|
| 12 | Method B | 3 | Calibration level 1 and report |
| 13 | Method B | 5 | Calibration level 2 and report |
| 14 | Method B | 5 | Calibration level 2 and report |
| 15 | Method B | 20 | Sample analysis and report |
| 16 | Method B | 21 | Sample analysis and report |
| 17 | Method B | 22 | Sample analysis and report |
| 18 | Method B | 3 | Calibration level 1 and report |
| 19 | Method B | 5 | Calibration level 2 and report |
| 20 | Method B | 5 | Calibration level 2 and report |
| 21 | Method B | 23 | Sample analysis and report |
| 22 | Method B | 24 | Sample analysis and report |

Note that the results shown in Table 17 on page 187and  Table 18 on page 188can be obtained by using Partial Sequence to see a preview of the run order after setting up the Sequence Table.

# Explicit and Cyclic Calibrations Together

This type of sequence comprises explicit and cyclic calibrations in the same sequence.

This feature allows you to recalibrate the method completely at the beginning of a sequence (*explicit recalibration*) and then update the calibration (*cyclic recalibration*) during the sequence.

- *Two calibration lines for each calibration level in the Sequence table must be specified.* One calibration line is for the explicit recalibration entry and the other for the cyclic recalibration entry.

- The sequence table *must* have entries for each calibration line and all cyclic recalibration vials *must* appear before both the explicit recalibration and sample entries.

## Example

The sequence table below illustrates a single-level calibrated method called SimpReg. It is truncated to simplify the example.

**Table 19**    Sequence Table for SIMPREG

| 1 | 1 | SimpReg | 1 | Calibration | 1 | Average | Average | 3 |
|---|---|---------|---|-------------|---|---------|---------|---|
| 2 | 1 | SimpReg | 1 | Calibration | 1 | Replace | Replace | |
| 3 | 2 | SimpReg | 1 | | | | | |
| 4 | 3 | SimpReg | 1 | | | | | |
| 5 | 4 | SimpReg | 1 | | | | | |
| 6 | 5 | SimpReg | 1 | | | | | |
| 7 | 6 | SimpReg | 1 | | | | | |

There are two entries for the single calibration level.

- The first calibration line is for the same level but averages the calibration parameters. The interval entry specifies that the recalibration is done after every three samples.

- The second entry replaces all the recalibration parameters, that is, a complete recalibration is done. It has *no* recalibration interval.

## Sequence Table

The sequence table comprises seven lines. The first line specifies the cyclic recalibration sample. The second line specifies the explicit recalibration that is done only once at the beginning of the sequence. The third through seventh lines specify the samples to be analyzed.

The order of the entries into the sequence table is very important. All cyclic recalibration vial entries specifying cyclic calibration *must* appear *before* the sample entries or any explicit recalibration entries for the method.

# SimpReg Analysis Order

This section describes the analysis order for the SimpReg method.

**Table 20**    SimpReg Analysis Order

| Seq Line | Inj No. | Method | Vial | Operation |
|----------|---------|--------|------|-----------|
| 2 | 1 | SimpReg | 1 | Simple calibration |
| 1 | 2 | SimpReg | 1 | Regular calibration |
| 3 | 3 | SimpReg | 2 | Sample analysis |
| 3 | 4 | SimpReg | 3 | Sample analysis |
| 4 | 5 | SimpReg | 4 | Sample analysis |
| 5 | 6 | SimpReg | 1 | Regular calibration |
| 6 | 7 | SimpReg | 5 | Sample analysis |
| 7 | 8 | SimpReg | 6 | Sample analysis |

# Cyclic Calibration Sequences with Bracketing

In a cyclic calibrated sequence with bracketing, the calibration table used to calculate the unknown quantitative results is generated by averaging the results of the current calibration with those of the previous calibration. This new calibration table is a more accurate representation of the instrument response at the time the sample was analyzed.

## Example

Consider the following situation:

- The instrument response is drifting.
- Three injections of an identical two-component mixture are specified.
- Two injections are specified as calibration samples, and one is specified as a sample.
- The first and third are calibration samples.
- The second injection is a sample.

To obtain a precise quantitative result for injection two (the sample), an interpolation must be made between the two calibration samples; see the figure. This process is known as bracketing.
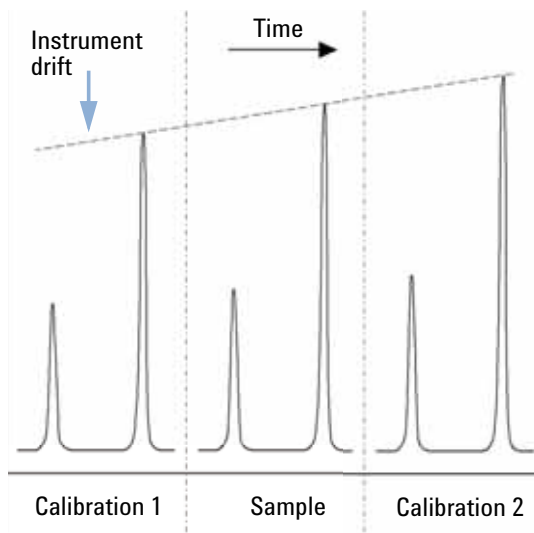
**Figure 41**    Bracketing

## Bracketed Sequence Operation

- The first calibration vials are analyzed.
- The sample vials are analyzed.
- The next calibration vials are analyzed.
- The calibration table is produced by replacing the existing response factors by new ones and averaging the following calibration runs into a new calibration table.
- The sample vial data files are evaluated, and reports are generated.
- The sequence returns to step 2 if there are more sample vials to be analyzed.

## Example

This section describes a sequence bracketing example comprising one method called Brack.M. The Brack.M method is a two-level internal standard method using cyclic calibration.

## Sequence Table

The sequence table of Brack.M (next page) is truncated to simplify the example. It consists of seven lines. The first two lines define the recalibration conditions for each level. The remaining lines define the samples to be analyzed.

More specifically, the sequence table of Brack.M method has:

- A Bracket entry in the Update Response Factor column that specifies brakketing of samples with calibration samples.
- A Replace entry in the Update Retention/Migration Times column which specifies a replacement of the retention/migration times.
- An entry of 3 in the Recalib Interval column that specifies recalibration after every three samples.

**Table 21**    Sequence Table for BRACK-M

| 1 | 1  | BRACK-M | 2 | Calibration | 1 | Bracket | Replace | 3 |
|---|----|---------|---|-------------|---|---------|---------|---|
| 2 | 2  | BRACK-M | 2 | Calibration | 2 | Bracket | Replace | 3 |
| 3 | 10 | BRACK-M | 1 |             |   |         |         |   |
| 4 | 11 | BRACK-M | 1 |             |   |         |         |   |
| 5 | 12 | BRACK-M | 1 |             |   |         |         |   |
| 6 | 13 | BRACK-M | 1 |             |   |         |         |   |
| 7 | 14 | BRACK-M | 1 |             |   |         |         |   |

## Bracketed Sequence Analysis Order

```
---------------------------------------------------------------------------
Run   Method    Vial Inj  DataFile    Lvl  Upd  Upd  Operation
No.   Name      No.  No.   Name        No.  RF   Ret
---------------------------------------------------------------------------
 1    Brack.M    1    1    c1-03001.d   1    R    R   Report for Calibration Run No.1
 2    Brack.M    1    2    c1-03002.d   1    A    R   Report for Calibration Run No.2
 3    Brack.M    2    1    c2-03001.d   2    R    R   Report for Calibration Run No.3
 4    Brack.M    2    2    c2-03002.d   2    A    R   Report for Calibration Run No.4
                                                      Print Calibration Table
 5    Brack.M   10    1    010-0301.d                 Sample Analysis, no report
 6    Brack.M   11    1    011-0301.d                 Sample Analysis, no report
 7    Brack.M   12    1    012-0301.d                 Sample Analysis, no report
 8    Brack.M    1    1    c1-03003.d   1    A    R   Calibration Analysis, no report
 9    Brack.M    1    2    c1-03004.d   1    A    R   Calibration Analysis, no report
10    Brack.M    2    1    c2-03003.d   2    A    R   Calibration Analysis, no report
11    Brack.M    2    2    c2-03004.d   2    A    R   Calibration Analysis, no report
                                                      Print Calibration Table
                         010-0301.d                 Report for   Sample      Run No.5
                         011-0301.d                 Report for   Sample      Run No.6
                         012-0301.d                 Report for   Sample      Run No.7
                         c1-03003.d   1    R         Report for Calibration Run No.8
                         c1-03004.d   1    A         Report for Calibration Run No.9
                         c2-03003.d   2    R         Report for Calibration Run No.10
                         c2-03004.d   2    A         Report for Calibration Run No.11
12    Brack.M   13    1    013-0301.d                 Sample Analysis, no report
13    Brack.M   14    1    014-0301.d                 Sample Analysis, no report
14    Brack.M    1    1    c1-03005.d   1    A    R   Calibration Analysis, no report
15    Brack.M    1    2    c1-03006.d   1    A    R   Calibration Analysis, no report
16    Brack.M    2    1    c2-03005.d   2    A    R   Calibration Analysis, no report
17    Brack.M    2    2    c2-03006.d   2    A    R   Calibration Analysis, no report
                                                      Print Calibration Table
                         013-0301.d                 Report for Sample Run No.12
                         014-0301.d                 Report for Sample Run No.13
                         c1-03005.d   1    R         Report for Calibration Run No.14
                         c1-03006.d   1    A         Report for Calibration Run No.15
                         c2-03005.d   2    R         Report for Calibration Run No.16
                         c2-03006.d   2    A         Report for Calibration Run No.17
---------------------------------------------------------------------------
                      Where A = average
                            R = replace
```

# Cyclic Recalibration Sequences with Multiple Vials Containing the Same Dilution of a Standard

## Cyclic Recalibration Sequence with "Round-Robin" Calibration Vial Usage

When running a large sequence that performs cyclic recalibrations, that is, performs an automatic recalibration after a fixed number of sample injections, there is a potential risk of emptying the volume of calibration vial during the course of the sequence. The ChemStation sequence table provides a means of using a series of vials that contain the same dilution of a standard to be used in a *round-robin* fashion.

With this capability, large sequences can be defined with automatic recalibrations at fixed intervals using multiple calibration vials for each level and each calibration vial would be consumed to the same extent.

By defining an appropriate number of calibration vials, it is even possible to guarantee that each calibration vial is only used once. This is an important requirement in cases where a fresh calibration vial is required for every recalibration, for example, because the analyte evaporates once the septum is punctured or starts degrading after getting in contact with the steel needle. The following section describes how the ChemStation sequence table needs to be set up to fulfill these requirements.

Determine the total number of calibration vials for each level based on the estimated calibrant usage throughout the sequence.

Set up a separate cyclic recalibration line for each calibration vial. Lines defined for the same calibration level must be in adjacent sequence lines and the defined vial positions must be adjacent, too. Choose an identical recalibration interval for all calibration lines. For example, if your sequence should recalibrate after every 6 sample injections, set the recalibration interval to 6.

**Table 22**    Cyclic Recalibration Sequence with 3 Vials Defined for Each Level

| Vial No. | Sample Name | Sample Type | Method Name | No. of Inj. | Lvl | Upd RT | Upd RF | Interval |
|----------|-------------|-------------|-------------|-------------|-----|--------|--------|----------|
| 1 | Cal1a | Calib | MethodA | 1 | 1 | Avg | Avg | 6 |
| 2 | Cal1b | Calib | MethodA | 1 | 1 | Avg | Avg | 6 |
| 3 | Cal1c | Calib | MethodA | 1 | 1 | Avg | Avg | 6 |
| 5 | Cal2a | Calib | MethodA | 1 | 2 | Avg | Avg | 6 |
| 6 | Cal2b | Calib | MethodA | 1 | 2 | Avg | Avg | 6 |
| 7 | Cal2c | Calib | MethodA | 1 | 2 | Avg | Avg | 6 |
| 10 | Sample10 | Sample | MethodA | 6 | | | | |
| 11 | Sample11 | Sample | MethodA | 6 | | | | |
| 12 | Sample12 | Sample | MethodA | 6 | | | | |
| 13 | Sample13 | Sample | MethodA | 6 | | | | |
| 14 | Sample14 | Sample | MethodA | 6 | | | | |

The execution order is:

- Vial 1 (Cal1a)
- Vial 5 (Cal2a)
- 6 injections out of vial 10 (Sample10)
- Vial 2 (Cal1b)
- Via 6 (Cal2b)
- 6 injections out of vial 11 (Sample11)
- Vial 3 (Cal1c)
- Vial 7 (Cal2c)
- 6 injections out of vial 12 (Sample12)
- Vial 1 (Cal1a)
- Vial 5 (Cal2a)
- 6 injections out of vial 13 (Sample13)
- Vial 2 (Cal1b)
- Vial 6 (Cal2b)
- etc.

## Cyclic Recalibrations Where Each Calibration Uses a Different Vial

To ensure that every calibration vial be injected only once, the sequence must define a sufficient number of different calibration vials, so that the *round-robin* order described in the previous example is not applied. For example, if the sequence processes 80 sample vials with recalibrations required after every 10 samples, the sequence table must contain 80/10 + 1= 9 calibration lines for each level.

As in the previous example, the calibration lines must be adjacent sequence lines that reference adjacent vial positions.

## Bracketing Sequence that Uses Different Vials for Opening and Closing Bracket

The same capability is available for bracketing sequences. By defining the appropriate vial range of calibration vials, a bracketing sequence can be defined so that different calibration vials are used for the opening and closing brackets. In this case, too, calibration lines in the sequence must be adjacent just like the vial positions of the calibration vials.

Whether or not the bracketing calibration vials are used in round-robin mode or for one single injection only simply depends on the total number of calibration vials for each level and the number of recalibrations required by the sequence.

The following example defines 3 sample injections that are bracketed by calibrations. The opening bracket uses a different calibration vial than the closing bracket. Recalibrations are required after every sample injection. so the recalibration interval has to be 1. The number of calibration lines per level is the number of samples plus one.

**Table 23** Different Vials Used for Opening and Closing Brackets

| Vial No. | Sample Name | Sample Type | Method Name | No. of Inj. | Lvl | Upd RT | Upd RF | Interval |
|----------|-------------|-------------|-------------|-------------|-----|--------|--------|----------|
| 1 | Cal1a | Calib | MethodA | 1 | 1 | Brkt | Brkt | 1 |
| 2 | Cal1b | Calib | MethodA | 1 | 1 | Brkt | Brkt | 1 |
| 3 | Cal1c | Calib | MethodA | 1 | 1 | Brkt | Brkt | 1 |

**Table 23**    Different Vials Used for Opening and Closing Brackets

| Vial No. | Sample Name | Sample Type | Method Name | No. of Inj. | Lvl | Upd RT | Upd RF | Interval |
|----------|-------------|-------------|-------------|-------------|-----|--------|--------|----------|
| 4 | Cal1d | Calib | MethodA | 1 | 1 | Brkt | Brkt | 1 |
| 10 | Sample10 | Sample | MethodA | 1 | | | | |
| 11 | Sample11 | Sample | MethodA | 1 | | | | |
| 12 | Sample12 | Sample | MethodA | 1 | | | | |

The execution order for this sequence is:

- Vial 1 (Cal1a), Opening Bracket 1
- Vial 10 (Sample10)
- Vial 2 (Cal1b), Closing Bracket 1 and Opening Bracket 2
- Vial 11 (Sample11)
- Vial 3 (Cal1c), Closing Bracket 2 and Opening Bracket 3
- Vial 12 (Sample 12)
- Vial 4 (Cal1d), Closing Bracket 3

# 9

# Data Review, Reprocessing and Batch Review

This chapter describes the possibilities to review data and how to reprocess sequence data. In addition it describes the concepts of Batch Review, Batch configuration, review functions, and batch reporting.

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# Navigation Table in Data Analysis

The Data Analysis view includes a Navigation Table that is designed to facilitate navigation through data files. The Navigation Table shows the runs contained in a selected data or sequence data subdirectory. You can use the Navigation Table to load access individual runs, or to automatically scroll through the loaded signals. For more details, please refer to the "Getting Started With New ChemStation Workflow" manual.

## Navigation Table Configuration

The Navigation table shows the data file information depending on the available data sets. The Navigation Table is read-only and the values in the Navigation table cannot be overwritten.

**Table 24**    Navigation Table Columns

| Single Runs Columns | Sequence Runs Columns |
| --- | --- |
| Overlay | Overlay |
| Date / Time | Line |
| Operator | Inj (Injection) |
| Vial | Vial |
| Data File | Sample Name |
| Sample Name | Method Name |
| Method Name | Sample Type |
| Manual Events | Manual Events |
| Sample Info | Cal Level (Calibration Level) |
| Sample Amount | Sample Info |
| ISTD Amount | Sample Amount |
| Multiplier | ISTD Amount |

**Table 24**    Navigation Table Columns

| Single Runs Columns | Sequence Runs Columns |
|---|---|
| Dilution | Multiplier |
| --- | Dilution |
| --- | Data File |

The Navigation table includes standard table configuration features, such as sorting and drag-and-drop options to move columns to different places. It is also possible to select the columns that are displayed in the Navigation Table.

In addition, column-specific grouping is possible, for example, single runs of a particular operator can be displayed by grouping the loaded files by the column "operator".

The Navigation table offers right mouse click functions to load a signal, overlay a signal, export data, print reports, view the acquisition method parameters etc. Each Navigation Table line can be expanded by clicking the + (plus) sign at the left of the line to configure signal-specific options:

- *Signal:* Lists the acquired signals and allows you to specify the signals to be loaded. The signal display selection is applied to each run individually.

- *General Info:* Lists the header details about the run.

- *Instrument curves:* Allows you to select the instrument data curves to be displayed along with the chromatogram/electropherogram and in the printout.

## Navigation Table Toolbar

The **Navigation Table** includes two toolsets that allow you either to review single run/sequence data, or to reprocess sequence data.

### Data Review Toolset

The review functionality of the Navigation Table allows you to step automatically or manually through the loaded signals. Depending on the selection specified in the **Preferences / Signal/Review** Options, the system can automatically integrate the signal and print a report for each file as it is loaded. The method applied to the data file is shown in the top menu.

### Sequence Reprocessing Toolset

The sequence reprocessing toolset is available only when a sequence acquired with ChemStation B.02.01 or higher is loaded that was acquired with **Unique Folder Creation** switched on. It is possible to start, stop and pause the reprocessing of the sequence. In addition, the toolbar gives access to the following dialog boxes in order to change parameter for reprocessing sequences and printing:

- **Sequence Table** (copy of the original *.s template, located in the sequence data container)
- **Sequence Parameters** dialog box
- **Sequence Output** dialog box
- **Sequence Summary Parameters** dialog box
- **Extended Statistic Parameters** dialog box
- **Save Current Sequence**
- **Print Current Sequence**

## Data Review Using the Navigation Table

Depending on your required workflow, you can review data in one of three ways:

**1** Review your sequence data using the individual method of each data file (sequence data B.02.01or higher): Use the option **Individual Method from Data File (DA.M)** in the **Preferences / Signal/Review Options** to have the system load the individual data analysis method (DA.M) stored with the data file before loading sequence data. As each line in the Navigation Table is accessed during the data review process, the linked DA.M for the selected data file is loaded and used for reviewing and generating the report. The method name

is visible in the Status Bar; the system adds from data file in brackets (from data file) to indicate that the loaded method is the individual method for the data file.

**2** Review your data using the sequence method: Use the option **Sequence Method** in the **Preferences / Signal/Review Options** to have the system load the sequence method corresponding to the current line of the Navigation Table. This method is loaded whenever a data file is loaded and is used for reviewing and generating the report. The method name is visible in the Status Bar; the system adds sequence in brackets (sequence) to indicate that the loaded method is the sequence method corresponding to the current line of the Navigation Table.

**3** Review your data using a different method: If you want to use a different method for reviewing the data than the individual data analysis method (DA.M) stored with the data file or the sequence method, the option **current method** in **Preferences / Signal/Review Option** must be selected. In this case, the system uses the currently loaded method for reviewing and generating the report. The method name is visible in the Status Bar.

| NOTE | For LC, CE, LC/MS and CE/MS systems, the option **Individual Method from Data File (DA.M)** is selected by default. |
|------|--------|

For GC systems, the option **Current Method** is selected by default.

## Sequence Reprocessing Using the Navigation Table

| NOTE | Sequence data acquired with ChemStation revisions up to B.01.03 need to be reprocessed using the **reprocess** option in the **Method and Run Control** view. The same applies to data acquired in B.03.01 when Unique Folder Creation is switched off. |
|------|--------|

Sequence data acquired with ChemStation revisions B.02.01 and higher need to be reprocessed using the reprocessing toolset in the **Data Analysis** Navigation Table.

For reprocessing using the Navigation Table in **Data Analysis**, all necessary files are present in the sequence data container:

- sequence data files (*.d)
- all methods (*.m) files used during the sequence
- copy of the original sequence template (*.s)

- sequence-related batch (*.b) file

- sequence-related logbook (*.log) file

During reprocessing, the individual methods DA.M for the data files are updated as well as the batch file (*.b) file.

With the **Data Analysis** reprocessing functions, it is possible to modify the sequence template (*.s) in the data container in order to change the multiplier, dilution etc., or to use a different method for reprocessing. By default, the Data Analysis reprocessing sequence parameter **parts of method to run** is set to **Reprocessing only**, and the option **Use Sequence Table Information** is checked. These predefined default values enable you to change the parameters in the sequence template and run a reprocess without editing the **Data Analysis** sequence parameters again.

If you have not explicitly changed the method in the sequence template, the system uses the sequence methods stored in the sequence data container to reprocess the sequence. These methods are the original methods used during data acquisition. Note that, even if the option **load DA method from data file** in **Preferences / Signal/Review Options** is selected, the system uses the sequence container method for reprocessing, not the DA.M of the individual data files.

If particular method parameters need to be changed (for example, specify to print to a *.xls file), the methods in the sequence container need to be modified and saved. This general change is then applied to all data files during reprocessing.

If you now want to use the updated sequence container method for further data acquisition, you need to copy this method from the sequence data container to one of the defined method paths. The new/updated method is then available in the ChemStation Explorer in the method view as a master method.

# What is Batch Review?

Batch review is a capability within data analysis designed to help an analyst perform a "first-pass" review of the results of a sequence or a selection of runs quickly and easily. It will save time especially when reprocessing large numbers of samples. Whenever a sequence is run, a batch file (with a .b extension) is automatically generated and placed in the data directory along with the data files. This batch file contains pointers to the data files in the batch review itself. Upon loading a batch, the analyst need only select the method to use for the batch, and then individually select the desired data files to analyze in the batch. One can check the calibration accuracy, instrument performance and individual integrations before approving the results. Any chromatogram specific integration parameters which are modified can be stored with the data file for data traceability. This interactive environment provides complete access to all other data analysis capabilities, such as peak purity, library searching, etc., as well.

Batch review uses the same data analysis registers (ChromReg and ChromRes) as the standard data analysis and should therefore not be used in an online session that is currently performing analyses.

# Enabling Batch Review Functionality with ChemStation OpenLAB Option Installed

When ChemStation OpenLAB Option is installed, the Batch Review functionality is not available by default. In order to use Batch Review, this functionality has to be enabled by an entry in the [PCS] section of the ChemStation.ini file. The file is located in the windows directory c:\ WINDOWS.

**[PCS]**

**_BatchReview=1**

The default entry, _BatchReview=0, turns off the functionality.

# Batch Configuration

A batch is a user-selected series of data files that is processed using a user-defined method. All data files in the batch are processed using the same method. The processing steps carried out each time a new sample is loaded for review can be selected (integration, identification/quantitation, reporting).

All calibration runs in the batch are used to produce a single calibration table, using averaged response factors, which is then used for quantification.

## Batch Table

Runs are displayed in a user-configurable batch table:

- the number and content of the table's columns can be specified;
- the runs can be sorted by
  - run index (the order in which the runs were acquired) independent of any other criteria,
  - sample type (control samples first, then calibration samples, then normal samples) then by run index within each sample type,
  - method (if more than one method was used to acquire the runs) then by run index within each method;
- samples, calibration samples and control samples can be displayed in the table or hidden.

Each selected run occupies a line in the batch table. You can exclude a run in the batch table (e.g. from calibration) by changing its sample type to Removed.

## Compound Table

The compound results are displayed in a user-configurable compounds table, but contents of the compounds table depends on the type of samples in the batch table:

- the compound list contains all compounds found in the method that was loaded for batch review.

- if calibration samples only are displayed in the batch table (samples and control samples are hidden), the compound table shows additional columns for calibration-related information (expected amount, relative error and absolute error).

- if control runs only are displayed in the batch table (samples and calibration samples are hidden), the compound table shows additional columns for any defined control limits.

For columns containing compound-specific information, you can include the name of the compound in the table title by adding %s to the column specification.

## Batch Report

The batch report contains two tables that are generally analogous to the batch table and the compound table; these tables are also user-configurable.

For columns containing compound-specific information, you can include the name of the compound in the table title by adding %s to the column specification. Multi-line headers are allowed; you insert the character '|' at the point where you want the line to break.

## User Interface

Batch review provides a choice of two user interfaces:

- the standard interface includes a button bar, with buttons mirroring most of the Batch menu items, together with the batch table and compound table;

- a minimal interface provides a button bar similar to the standard interface, but replaces the batch table and compound table with a combo box that contains only the information specified for the batch table. The minimal interface button bar does not contain batch table-related or compound table-related buttons.

# Review Functions

Data files can be displayed in one of two ways:

- manually, by selecting a run to display from the table,

- automatically, with a predefined interval between each data file. During automatic display, only those sample types displayed in the table are displayed; the runs are displayed in the order in which they appear in the table. The automatic review can be paused and later resumed, or stopped.

The standard functions provided by the ChemStation are available with batch review. This includes calibration, manual manipulation of chromatograms, for example by smoothing or manual integration. Any changes made to a data file can be marked and saved with the batch file. Chromatograms that have been reviewed are marked with an asterisk in the batch table. You can also discard changes made to either the current chromatogram only, or all changes to all chromatograms in the batch.

When a run is loaded, the selected processing options are performed; if the run has already been processed and the changes saved, the processed run is loaded. This is a faster process than loading the unprocessed run, because no processing needs to be done.

## Calibration in Batch Review

Calibration in batch review works independently from the recalibration settings in the sequence table. The first step in batch calibration always replaces both response and retention time entries in the calibration table. For the following calibration standards, both response and retention time values are averaged.

# Batch Reporting

The user-configurable "Batch Table" on page 209, can be printed directly on the printer, displayed on the screen or printed to a file with a user-specified prefix in one of the following formats:

- UNICODE text file with the extension .TXT
- Data Interchange Format with the extension .DIF
- Comma-Separated Values format with the extension .CSV
- Microsoft Excel format with the extension .XLS.

The reporting options also give the possibility of sorting the samples (by Run Index, Sample Type or Method) independent of the sort method in the batch table. The sort priorities are as for the "Batch Table" on page 209.

## Batch History

Batch review maintains a log of all actions relating to the current batch. Any action that changes the batch (for example changing the displayed chromatogram, changing the sample type, loading and saving the batch) adds a line to the batch history with a date and time stamp, the current operator name and a description of the event.

You can also add your own comments to the batch history. Existing batch history entries cannot be edited, and the history list cannot be accessed except through the Batch History menu item.

# 10
# Using the ChemStation Reports

This chapter describes what a report is. It gives details on reporting results, quantitative results, report styles, report destination, and sequence summary reporting.

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# What is a Report?

A report can comprise quantitative and qualitative information of the sample you analyze. The report can be a hardcopy printout, a display on the screen or an electronic file. The report can include details of the peaks detected during the run and plots of the signals that were acquired.

# Reporting Results

Two types of reports are available:

- an uncalibrated report which does not correct for detector response, and
- a calibrated report that shows results corrected for the difference in the detector response to various components of the sample.

## Uncalibrated Reports

The uncalibrated reports include the **Area%** and **Height%** reports. These reports are mainly used in the preparation of calibrated reports. They may be of value as a final report if the amounts of compound required to produce a unit area or height response for the compounds of interest are similar.

## Calibrated Reports

Calibrated reports correct for the difference in the detector response to the reported compounds. One or more calibration samples containing known amounts of the reported compounds must be run under the same conditions used for the unknown sample. The integration data from these calibration sample(s) are used to prepare a calibration table. This is a list of retention/migration times, amounts and responses which is used in the generation of the report. The calibrated reports are based on two calibration procedures called external standard and internal standard.

## External Standard Report

The ESTD report lists the results with the units of your choice or with each compound as a percentage of all the compounds present. The external standard procedure requires that the relative injected volume of both the

calibration and unknown samples must be known accurately. The reliability of the external standard report is limited by the reproducibility of the injection and any other factors that might change from sample to sample.

## Internal Standard Report

The limitations of the external standard procedure can be overcome by using the internal standard approach. An accurately known amount (not necessarily the same amount) of the internal standard is added to both the calibration sample(s) and unknown sample. The response of each compound of interest is divided by the response of the internal standard to give a response ratio. The calibration curves are a plot of this response ratio versus the amount ratio and this information is used in the calculation of the reported results. In this way inadvertent errors in injection volume or slight changes in the chromatographic/electropherographic system that affect all compounds similarly are cancelled out. The ISTD report lists the results with units of your choice.

## Control Charts Report

The Control Charts Report tracks a single result from multiple runs for a specific calibrated compound. The **Control Chart** feature is installed after the ChemStation is operational. Methods that use this feature pass the tracked result to a Microsoft Excel work sheet after each run. Excel is then used to print the report.

# Quantitative Results

The report type is identified by the name of the calculation method used to prepare it, for example, an ISTD report. Each type is briefly described below. The calculations for each report are given in "Quantitative Results" on page 217.

**Area%** provides the simplest report and requires no calibration data since no correction is made for the difference in detector response of sample components. The Area% report is particularly useful in developing a calibration table for use with the other report options. This report is suitable for analyses in which the difference in detector response of the components is not significant.

**Height%** gives a report similar to the Area% report except that peak height is used for the calculations instead of peak area.

**Norm%** gives a report in which each component is reported as a percentage of all the components present. The peaks are corrected for detector response before calculation of the percentage of each.

**ESTD** produces a report of the actual amount of each substance in whatever units you choose. Amounts are calculated using a previously established calibration table. The use of an external standard requires that the injected volume of the calibration mixture be known.

**ESTD%** produces a report of the relative amount of each substance as a percentage of the injected sample. Amounts are calculated using a previously established calibration table. The use of an external standard requires that the injected volume of the calibration mixture be known.

**ISTD** produces a report of the actual amount of each substance. Amounts are calculated using a previously established calibration curve. The use of an internal standard in both the sample and the calibration mixture eliminates the need to know and control the volume of the sample injected. This also corrects for any variation in instrument performance between runs.

**ISTD%** produces a report of the relative amount of each substance as a percentage of the injected sample. The use of an internal standard in both the sample and the calibration mixture eliminates the need to know and control the volume of sample injected. This also corrects for any variation in instrument performance between runs.

# Reporting Custom Field Values

The values of the custom fields attached to a certain sample according to its acquisition method can be added to the report. Sample custom fields are listed at the end of the report header that contains the general sample information. Compound custom fields appear at the end of the report.

# Report Styles

The following report styles are available:

You choose to add a signal to any of the report styles by checking the appropriate box in the Specify Report dialog box.

- **None** - No text will be reported. The chromatogram will be reported only if the Add Chromatogram Output option is selected.
- **Short** - Comprises quantitative text results of all integrated signals which are setup in the Signal Details dialog box (LC only) or the Signal dialog box (GC only). The peak width in the short report is calculated with the more complex formula used by the integrator: $PW = 0.3(IPRight - IPLeft) + 0.7(Area/Height)$ where IPRight and IPLeft are the inflection points.
- **Detail** - Comprises header, quantitative results, and calibration curves. The header is stored in a file called RPTHEAD.TXT in the method directory. You can change the header using a text editor to include a method specific text.
- **Header** + **Short** - Comprises file header and quantitative text results. The header is stored in a file called RPTHEAD.TXT in the method directory. You can change the header using a text editor to include a method specific text.
- **GLP** + **Short** - Comprises header, sample information, instrument conditions, logbook, signal, and quantitative results. The header is stored in a file called RPTHEAD.TXT in the method directory. You can change the header using a text editor to include a method specific text.
- **GLP** + **Detail** - Comprises header, sample information, instrument conditions, logbook, signal, quantitative results, and calibration curves. The header is stored in a file called RPTHEAD.TXT in the method directory. You can change the header using a text editor to include a method specific text.
- **Full** - Comprises header, sample information, instrument conditions, logbook, signals, and quantitative results. The header is stored in a file called RPTHEAD.TXT in the method directory. You can change the header using a text editor to include a method specific text.
- **Performance** - Produces a report according to the limits specified in the Edit Performance Limits dialog box of the System Suitability menu.

For uncalibrated methods, the report parameters include the peak number, retention/migration time, peak area, peak height, signal description, true half-height peak-width (see also "True Peak Width Wx [min]" on page 246), symmetry, k', efficiency (plates) and resolution for each peak.

For calibrated methods, the report parameters include the peak number, retention/migration time, compound name, amount, signal description, true half-height peak-width, symmetry, k', efficiency (plates) and resolution for each peak.

The half-height peak calculation is not the same as the more complex peak width formula used by the integrator. The values of efficiency and resolution are based on this calculated peak width. The report header consists of all method relevant information, including instrument, column/capillary, sample, and acquisition parameters. The signal is also plotted.

- **Performance** + **Noise**- Combines the Performance report style with the noise calculations for the noise ranges defined in the Edit Noise Range dialog box of the System Suitability menu. In addition, the noise, given as six times the standard deviation, as peak-to-peak, and as ASTM noise.The drift and wander is also determined.

- **Performance** + **Extended**- Produces an extended report with all the parameters from the peak performance calculations and individual plots of each peak. The plots include the baseline, the tangents, and the peak widths at defined heights. This report type includes only calibrated peaks.

In addition to the parameters printed for the Performance report style, more Peak Performance Parameters are determined: the peak start and end peak times, skew, excess, peak width, USP tailing factor, time interval between data points, number of data points, statistical moments, plates, plates per meter, selectivity and resolution of each peak are printed. The peak width, plates, plates per meter, selectivity, and resolution are calculated by the true half height, 5 sigma, tangent and tailing methods (please refer to "Performance Test Definitions" on page 244 for details).

The report header consists of all method relevant information such as instrument, column/capillary, sample and acquisition parameters and a plot of the signal. For a complete list of the peak performance parameter algorithms see "Performance Test Definitions" on page 244.

The spectral report styles (**Short** + **Spectrum**, **Detail** + **Spectrum**, **Performance** + **Library Search**) are described in *Understanding Your Spectra Module*.

## Adding a Customized Report to Report Styles

You can add a custom report template that you have created in the Report Layout view of the ChemStation to the list of available report styles.

| NOTE | All reports apart from the performance reports list the peakwidths calculated with a more complex formula by the integrator (for details on the peakWidth calculation please refer to "Peak Width" on page 80). |
|------|------|

# Other Report Style Parameters

## Summed Peaks Table

The peak sum table is provided for certain applications in the petrochemical and pharmaceutical industries that can be performed more effectively with the following features:

- Sum the areas of peaks that lie within a range specified by the user
- Sum the areas of a range of peaks and perform the calculations using a single multiplier
- Sum the areas of all peaks having the same name

When the report is generated, the ChemStation uses the peak sum table to produce a peak sum report that is printed after the standard report calculations with the exception of Norm% which is replaced by the peak sum report.

## Report Layout for Uncalibrated Peaks

To change the report layout for uncalibrated peaks, choose one of the following in the Specify Report dialog box.

- Separately to report the uncalibrated peaks in a separate table if sorting by retention/migration time is selected, or separate tables if sorting by signal is selected.
- With Calibrated Peaks to report the uncalibrated peaks together with the calibrated peaks.
- Do Not Report to suppress the reporting of uncalibrated peaks.

# Report Destination

The report can be sent to either:

- Screen

  The report (including text and graphics) is displayed on the screen in the report preview window from which it can be printed.

- Printer

  The report comprising text and graphics is printed on the currently selected printer.

- File

  The report is saved to a file. If the data is saved to a file it is possible to reprocess the data with another program, for example, Microsoft Windows EXCEL.

## Report File Formats

A report can be saved in different formats. Each format has a specific extension. It is possible to select more than one format for a report.

**.TXT**    The report text is printed as a UNICODE text file.

**.EMF**    Each report graphic (signal or calibration curve) is saved in a Microsoft Windows metafile (WMF). Several .WMF files for one report are possible. The generated file format adheres to the Microsoft standard metafile format as defined in the Windows software development documentation. These files are compatible with the Aldus Placeable Metafile (APM) format used by a number of proprietary software packages.

**.DIF**    The tabular report data is saved in Data Interchange Format (DIF). This format is accepted by spreadsheet programs, for example, Microsoft Windows EXCEL. Independent from the report style selected, only the information contained in the report style Short will be saved.

**.CSV**    The report is in Comma Separated Values (CSV) format. This is a very simple format for tabular data that is accepted by many spreadsheet programs and databases. Independent from the report style selected, only the information contained in the report style "Short" will be saved.

There can be several .DIF and .CSV files for a single report. For each report block, the first file, for example, REPORT00.CSV, contains the report header information. Subsequent files contain the tabular results.

If the results are sorted by retention/migration time, only one file is required for the complete table, for example, REPORT01.CSV.

If the results are sorted by signal, a separate table is required for each signal. In this case, the files are named Report01.CSV through ReportNN.CSV, where NN is the number of the signal.

**.XLS**    The report is exported to a Microsoft Excel spreadsheet in (XLS) format. The data generally requires additional processing.

**.PDF**    The report is printed to a .pdf file. For the .pdf reports ChemStation uses a PDF printer, called "novaPDF Pro Server v5". The corresponding license for this printer is only valid for printing from within ChemStation. If you want to use this printer from other applications you have to buy a license for that purpose.

# Sequence Summary Reporting

## Overview

The ChemStation can print a variety of standard reports for individual sample analyses. Sequence summary reporting is an additional way of reporting, that allows you to calculate and report parameters across a number of different analyses. It is useful, for example, to test the stability of an instrument or the robustness of a new method.

A sequence summary report may include:

- a title page,
- instrument configuration, including the revision numbers of the instrument and details of the analytical column/capillary being used,
- sequence table listings which describe what the automated sequence of analyses should have done,
- logbook descriptions of what the sequence actually did and any unexpected events which occurred during the sequence,
- method listings,
- individual reports on each sample,
- statistics on the analyses based on selected criteria—s*tatistics are calculated only for calibrated compounds*, and
- a table of contents with page number references to the detailed sections of the report.

## Setting up a Sequence Summary Report

When setting up a sequence summary report, you can select any combination of the following nine categories by activating the appropriate check boxes and, where appropriate, selecting a report style from the template selections. Each template specifies the contents and layout of that particular section of the whole sequence summary report.

You can choose one of the following sequence summary report styles:

### One Page Header

The GLP template prints GLP in large letters as a title page for the following report. It also includes the date and a place for a signature.

### Configuration

Select Configuration if you want to include the instrument configuration and analytical column/capillary specifications in the report.

### Sequence Table

Select Sequence table to include a list of the samples, sample quantification parameters and method names in the report. This list shows what the system should have run.

### Logbook

Select Logbook for a listing of the analyses the system ran, including instrument conditions and any unusual events that occurred while the samples were being analyzed.

### Methods

Select Methods to list all the analytical methods used in the series of automated analyses.

### Analysis Reports

Select Analysis reports to get individual analysis reports according to the report style set up for the method.

Individual analytical reports may be printed after each analysis according to the report style specified for the method in question, in addition to the report sections specified in Sequence Summary Reporting. See "Sequence Output".

### SUILabel Type = Application > Statistics for Calibrated and Sample Runs

Selecting the Statistics cal. runs will produce statistical trend analyses for calibration samples. Selecting the Statistics sample runs will produce statistical trend analyses for sample (unknown) analyses. Both selections have Standard Statistic and Extended Statistic template styles. Extended Statistics

prints the statistical trends of the analyses as graphs, whereas the Standard Statistics selection prints only text. The selections that you make in the Items and Limits for Extended Statistics dialog box are used only when you choose the Extended Statistic option(s) in the Sequence Summary Parameters dialog box.

If you choose the Standard Statistic option(s) in the Sequence Summary Parameters dialog box, then the statistics reported are:

- retention/migration time,
- area,
- height,
- amount,
- peak width (based on the report style, see "Report Styles" on page 220)
- symmetry.

The statistic calculation does not distinguish between different calibration levels in a sequence that uses multilevel calibration methods. This means that the concentration-dependant items, for example, Area, Height, Amount (see the dialog box for Items and Limits for Extended Statistics) are all taken together, regardless of the calibration level. The Statistics for Calibration Runs values are therefore not useful for multilevel calibration methods in sequences.

### Summary

The Summary selection will print an overview of the series of samples analyzed and the methods used. If the Summary choice is selected together with other Sequence Summary selections, page numbers referring to the other parts of the sequence summary report are included. Two Summary styles are available:

The Sample Summary tabulates details of the sample analyses run in the sequence, with some sample information such as the sample name, data file name, method and vial number.

The Compound Summary tabulates the sample analyses with basic quantification results for each calibrated compound, or each peak, depending on the type of report specified in the method.

## Sequence Output

In the Sequence output dialog box you can also define where the sequence summary report should be printed.

Selecting Report to file and entering a file name prints the report to the specified file. The default setting is that data are saved to the file GLPrprt.txt. In GC systems with dual injection the data are saved in GLPrptF.txt and GLPrptB.txt for the front injector and back injector respectively.

Selecting Report to printer prints the report on the system's printer. Print individual reports for each run also activates printing of sample reports after each analysis. These reports are printed in addition to the reports specified for the sequence summary report which are produced at the end of the entire sequence. You can specify a new destination for these reports in the Sequence Output dialog or use the destination specified in the individual methods.

# 11
# Evaluating System Suitability

**Agilent Technologies**

**231**

This chapter describes what the ChemStation can do to evaluate the performance of both the analytical instrument before it is used for sample analysis and the analytical method before it is used routinely and to check the performance of analysis systems before, and during, routine analysis.

Evaluating the performance of both the analytical instrument before it is used for sample analysis and the analytical method before it is used routinely is good analytical practice. It is also a good idea to check the performance of analysis systems before, and during, routine analysis. The ChemStation software provides the tools to do these three types of tests automatically. An instrument test can include the detector sensitivity, the precision of peak retention/migration times and the precision of peak areas. A method test can include precision of retention/migration times and amounts, the selectivity, and the robustness of the method to day-to-day variance in operation. A system test can include precision of amounts, resolution between two specific peaks and peak tailing.

Laboratories which have to comply with:

- good Laboratory Practice regulations (GLP),
- good Manufacturing Practice regulations (GMP) and Current Good Manufacturing Practice regulations (cGMP), and
- good Automated Laboratory Practice (GALP).

Laboratories are advised to perform these tests and to document the results thoroughly. Laboratories which are part of a quality control system, for example, to comply with ISO9000 certification, will have to demonstrate the proper performance of their instruments.

The ChemStation collates results from several runs and evaluates them statistically in the sequence summary report.

The tests are documented in a format which is generally accepted by regulatory authorities and independent auditors. Statistics include:

- peak retention/migration time,
- peak area,
- amount,
- peak height,

- peak width at half height,
- peak symmetry,
- peak tailing,
- capacity factor (k´),
- plate numbers,
- resolution between peaks,
- selectivity relative to preceding peak,
- skew, and
- excess.

The mean value, the standard deviation, the relative standard deviation and the confidence interval are calculated. You can set limits for either standard deviation, the relative standard deviation or the confidence interval for each of these parameters. Should the values exceed your limits, the report is flagged to draw your attention to them.

The quality of the analytical data can be supported by keeping records of the actual conditions at the time the measurements were made. The ChemStation's logbook records instrument conditions before and after a run. This information is stored with the data and reported with sample data. Instrument performance curves are recorded during the entire analysis as signals, and stored in the data file. If supported by the instrument these records, overlaid on the chromatogram, can be recalled on demand, for example, during an audit.

Baseline noise and drift can be measured automatically. A minimum detectable level can be calculated from peak height data for each calibrated compound in the method.

Finally, instrument configuration, instrument serial numbers, column/capillary identification, and your own comments can be included in each report printed.

Extended performance results are calculated only for compounds calibrated for in the method, ensuring characterization by retention/migration times and compound names.

A typical system performance test report contains the following performance results:

- instrument details,

- column/capillary details,
- analytical method,
- sample information,
- acquisition information,
- signal description and baseline noise determination, and
- signal labeled with either retention/migration times, or compound names.

In addition, the following information is generated for each calibrated compound in the chromatogram:

- retention/migration time,
- $k'$,
- symmetry,
- peak width,
- plate number,
- resolution,
- signal-to-noise ratio, and
- compound name.

# Noise Determination

Noise can be determined from the data point values from a selected time range of a signal. Noise is treated in three different ways:

- as six times the standard deviation (sd) of the linear regression of the drift,
- as peak-to-peak (drift corrected), and
- as determined by the ASTM method (ASTM E 685-93).

The noise can be calculated for up to seven ranges of the signal; the ranges are specified as part of the system suitability settings in the reporting parameters.

## Noise Calculation Using Six Times the Standard Deviation
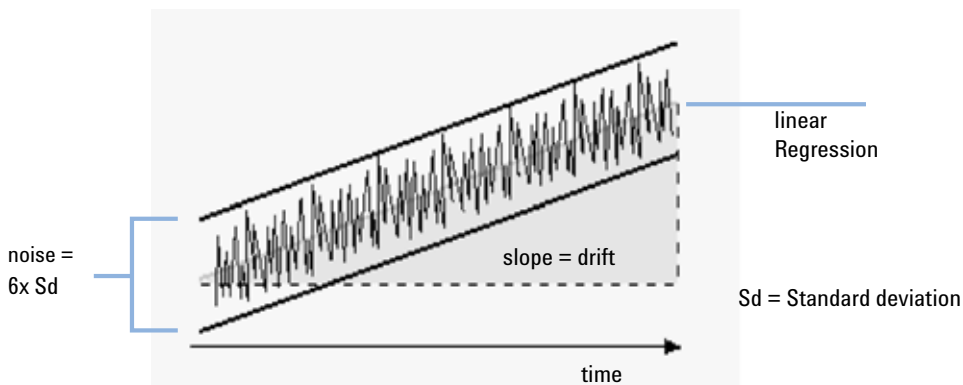


**Figure 42**    Noise as Six Times the Standard Deviation

The linear regression is calculated using all the data points within the given time range (see "Regression Analysis" on page 253). The noise is given by the formula:

$$N = 6 \times Std$$

where

N is the noise based on the six time standard deviation method, and

Std is the standard deviation of the linear regression of all data points in the time range.

# Noise Calculation Using the Peak-to-Peak Formula
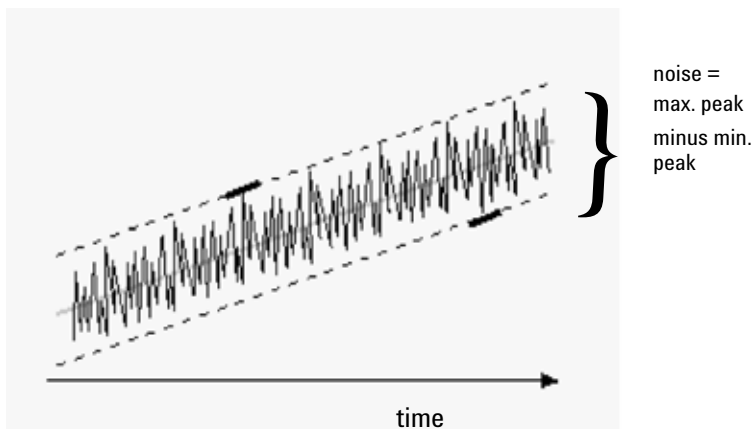


**Figure 43**     Noise as Maximum Peak to Minimum Peak (Distance)

The drift is first calculated by determining the linear regression using all the data points in the time range (see "Regression Analysis" on page 253). The linear regression line is subtracted from all data points within the time range to give the drift-corrected signal. The peak-to-peak noise is then calculated using the formula:

$N = I_{max} - I_{min}$

where

N is the peak-to-peak noise,

$I_{max}$ is the highest (maximum) intensity peak and

$I_{min}$ is the lowest (minimum) intensity peak in the time range.

# Noise Calculation by the ASTM Method
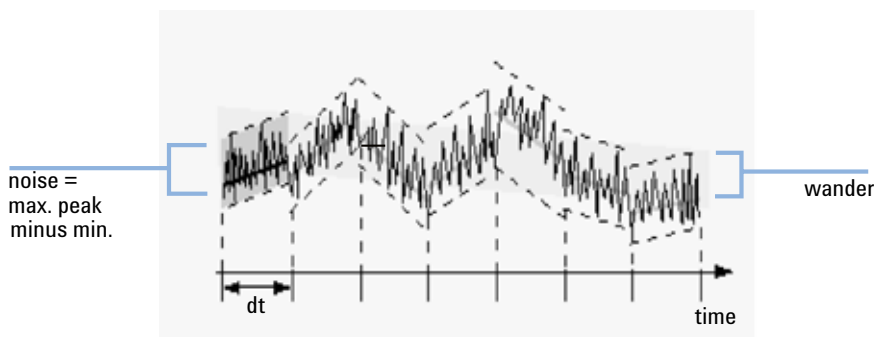


**Figure 44**    Noise Determined by the ASTM Method

ASTM noise determination (ASTM E 685-93) is based on the standard practice for testing variable-wavelength photometric detectors used in liquid chromatography, as defined by the American Society for Testing and Materials. Based on the size of the time range, three different types of noise can be distinguished. Noise determination is based on peak-to-peak measurement within defined time ranges.

## Cycle Time, t

*Long-term noise*, the maximum amplitude for all random variations of the detector signal of frequencies between 6 and 60 cycles per hour. Long-term noise is determined when the selected time range exceeds one hour. The time range for each cycle (dt) is set to 10 minutes which will give at least six cycles within the selected time range.

*Short-term noise*, the maximum amplitude for all random variations of the detector signal of a frequency greater than one cycle per minute. Short-term noise is determined for a selected time range between 10 and 60 minutes. The time range for each cycle (dt) is set to one minute which will give at least 10 cycles within the selected time range.

*Very-short-term noise (not part of ASTM E 685-93),* this term is introduced to describe the maximum amplitude for all random variations of the detector signal of a frequency greater than one cycle per 0.1 minute.

Very-short-term noise is determined for a selected time range between 1 and 10 minutes. The time range for each cycle (dt) is set to 0.1 minute which will give at least 10 cycles within the selected time range.

### Determination of the Number of Cycles, n

$$n = \frac{t_{tot}}{t}$$

where t is the cycle time and $t_{tot}$ is the total time over which the noise is calculated.

### Calculation of Peak-to-Peak Noise in Each Cycle

The drift is first calculated by determining the linear regression using all the data points in the time range (see "Regression Analysis" on page 253). The linear regression line is subtracted from all data points within the time range to give the drift-corrected signal. The peak-to-peak noise is then calculated using the formula:

$$N = \frac{I_{max}}{I_{min}}$$

where N is the peak-to-peak noise, $I_{max}$ is the highest (maximum) intensity peak and $I_{min}$ is the lowest (minimum) intensity peak in the time range.

### Calculation of ASTM Noise

$$N_{ASTM} = \frac{\sum\limits_{i=1}^{n} N}{n}$$

where $N_{ASTM}$ is the noise based on the ASTM method.

An ASTM noise determination is not done if the selected time range is below one minute. Depending on the range, if the selected time range is greater than, or equal to one minute, noise is determined using one of the ASTM methods previously described. At least seven data points per cycle are used in the calculation. The cycles in the automated noise determination are overlapped by 10 %.

## Signal-to-noise calculation

For the signal-to-noise calculation, the ChemStation uses the six times the standard deviation (sd) of the linear regression of the drift to calculate the noise. The range closest to the peak is selected from the ranges as specified in the system suitability settings. The signal-to-noise is calculated using the formula:

$$\text{Signal-to-Noise} = \frac{\text{Height of the peak}}{\text{Noise of closest range}}$$

The signal-to-noise is calculated for each peak in the signal. If the ChemStation cannot find a noise value, the signal-to-noise is reported as "-".

## Drift and Wander

Drift is given as the slope of the linear regression, see Figure Noise as Six Times the Standard Deviation, and wander is determined as the peak-to-peak noise of the mid-data values in the ASTM noise cycles, see Noise Determined by the ASTM Method.

# Calculation of Peak Symmetry

The ChemStation does not determine the asymmetry ratio of a peak, usually done by comparing the peak half-widths at 10% of the peak height, or 5% as recommended by the FDA.

Peak symmetry is calculated as a pseudomoment by the integrator using the following moment equations:

$$m_1 = a_1\left(t_2 + \frac{a_1}{1.5H_f}\right)$$

$$m_2 = \frac{a_2^2}{0.5H_f + 1.5H}$$

$$m_3 = \frac{a_3^2}{0.5H_r + 1.5H}$$

$$m_4 = a_4\left(t_3 + \frac{a_4}{1.5H_r}\right)$$

$$\text{Peak symmetry} = \sqrt{\frac{m_1 + m_2}{m_3 + m_4}}$$

If no inflection points are found, or only one inflection point is reported, then the peak symmetry is calculated as follows:

$$\text{Peak symmetry} = \frac{a_1 + a_2}{a_3 + a_4}$$
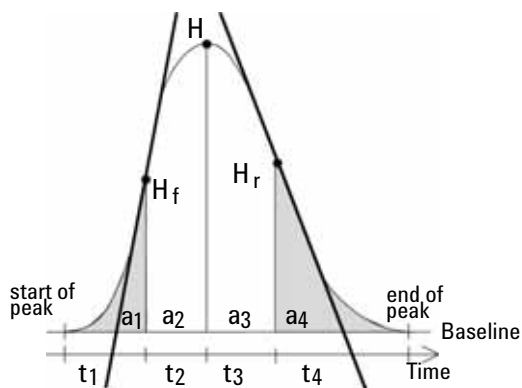
**Figure 45**    Calculation of the Peak Symmetry Factor

where:

$a_i$ = area of slice

$t_i$ = time of slice

$H_f$ = height of front inflection point

$H_r$ = height of rear inflection point

H = height at apex

# System Suitability Formulae and Calculations

The ChemStation uses the following formulae to obtain the results for the various System Suitability tests. The results are reported using the Performance, Performance + Noise and Performance + Extended report styles.

When ASTM or USP is specified for a given definition, then the definition conforms to those given in the corresponding reference. However, the symbols used here may not be the same as those used in the reference.

The two references used in this context are:

- *ASTM: Section E 682 – 93, Annual Book of ASTM Standards, Vol.14.01*
- *USP: The United States Pharmacopeia, XX. Revision, pp. 943 - 946*

# General Definitions

## Void Volume

$$V = d^2 \pi l (f/4)$$

where:

d = diameter of column [cm]

$\pi$ = constant, ratio of circumference to diameter of a circle

l = length of column [cm]

f = fraction of column volume that is not taken up by stationary phase but available for mobile phase; default value for f = 0.68 (for Hypersil)

## Retention Time of Unretained Compound t (m) [min]

(Also referred to as dead time or void time)

$$T_m = V/F$$

where:

F = flow rate of LC [ml/min]

# Performance Test Definitions

## Statistical Moments

$$M0 = d_t \cdot X$$

$$M1 = t_0 + d_t \cdot \frac{X}{Y}$$

$$M2 = \frac{d_t^2}{X} \cdot \sum_{i=1}^{N} \left( \left( i - 1 - \frac{Y}{X} \right)^2 \cdot A_i \right)$$

$$M3 = \frac{d_t^3}{X} \cdot \sum_{i=1}^{N} \left( \left( i - 1 - \frac{Y}{X} \right)^3 \cdot A_i \right)$$

$$M4 = \frac{d_t^4}{X} \cdot \sum_{i=1}^{N} \left( \left( i - 1 - \frac{Y}{X} \right)^4 \cdot A_i \right)$$

where:

| | |
|---|---|
| N | Number of area slices |
| $A_i$ | Value of area slice indexed by i |
| $d_t$ | Time interval between adjacent area slices |
| $t_0$ | Time of first area slice |
| $\sum_{i=1}^{N}$ | Sum of starting index 1 to final index N for discrete observations |

$$X = \sum_{i=1}^{N} (A_i)$$

$$Y = \sum_{i=1}^{N} ((i-1)A_i)$$

## Statistical Moments, Skew and Excess

Statistical moments are calculated as an alternative to describe asymmetric peak shapes. There is a infinite number of peak moments, but only the first five are used in connection with chromatographic peaks. These are called $0^{th}$ Moment, $1^{st}$ Moment, ... $4^{th}$ Moment.

The $0^{th}$ Moment represents the peak area.

The $1^{st}$ Moment is the mean retention time, or retention time measured at the center of gravity of the peak. It is different from the chromatographic retention time measured at peak maximum unless the peak is symmetrical.

The $2^{nd}$ Moment is the peak variance which is a measure of lateral spreading. It is the sum of the variance contributed by different parts of the instrument system.

The $3^{rd}$ Moment describes the vertical symmetry or skew. It is a measure of the departure of the peak shape from the Gaussian standard. The skew given additionally in the Performance & Extended report is its dimensionless form. A symmetrically peak has a skew of zero. Tailing peaks have positive skew and their 1. Moment is greater than the retention time. Fronting peaks have negative skew and their 1. Moment is less than the retention time.

The $4^{th}$ Moment or excess is a measure of the compression or stretching of the peak along a vertical axis, and how this compares to a Gaussian standard for which the 4. Moment is zero. It can be visualized by moving in or pulling apart the sides of a Gaussian peak while maintaining constant area. If the peak is compressed or squashed down in comparison, its excess is negative. If it is taller, its excess is positive. Also the excess is given in the Performance & Extended report in its dimensionless form.

# True Peak Width W$_x$ [min]

$$W_x = \text{width of peak at height x \% of total height}$$

| | |
|---|---|
| W$_B$ | base width, 4 sigma, obtained by intersecting tangents through the inflection points with the baseline (tangent peak width) |
| W$_{4.4}$ | width at 4.4% of height (5 sigma width) |
| W$_{5.0}$ | width at 5% of height (tailing peak width), used for USP tailing factor |
| W$_{50.0}$ | width at 50% of height (true half-height peak width or 2.35 sigma). |

# Capacity Factor (USP), Capacity Ratio (ASTM) k'

$$k' = \frac{T_R - T_0}{T_0}$$

where:

T$_R$ = retention time of peak [min]

T$_0$ = void time [min]

# USP Tailing Factor (USP) t

$$t = \frac{W_{5.0}}{t_w \cdot 2}$$

where:

t$_w$ = distance in min between peak front and TR, measured at 5% of the peak height

W$_{5.0}$ = peak width at 5% of peak height [min]
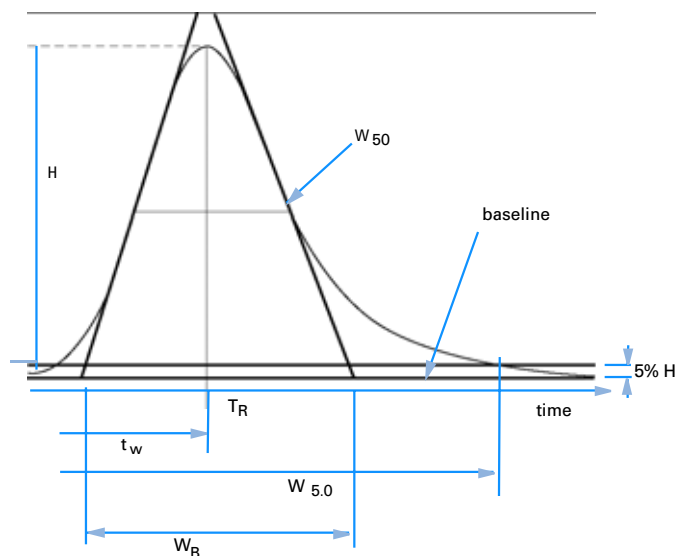
**Figure 46**     Performance Parameters

# Number of Theoretical Plates per Column (USP, ASTM) n

### Tangent method (USP, ASTM):

$$n = 16\left(\frac{T_R}{W_B}\right)^2$$

where:

$W_B$ = base width $[\min]$

### Half-width method (ASTM):

$$n = 5.54\left(\frac{T_R}{W_{50}}\right)^2$$

where:

$W_{50}$ = peak width at half-height $[\min]$

**5 Sigma method:**

$$n = 25\left(\frac{T_R}{W_{4.4}}\right)^2$$

where:

$W_{4.4}$ = peak width at 4.4% of peak height [min]

**Variance method:**

$$n = \frac{M1^2}{M2}$$

where:

Mx = x $^{th}$ statistical moment (see also "Statistical Moments" on page 244)

# Number of Theoretical Plates per Meter N [1/m]

$$N = 100 \times \frac{n}{l}$$

where:

n = number of theoretical plates

l = length of column [cm]

# Relative Retention (USP, ASTM), Selectivity Alpha

(Pertaining to peaks a and b, $T_R$ of peak a < $T_R$ of peak b)

$$\text{alpha} = \frac{k'_{(b)}}{k'_{(a)}}, \text{alpha} \geq 1$$

where:

$k'_{(x)}$ = capacity factor for peak x

# Resolution (USP, ASTM) R

(Pertaining to peaks a and b, $T_R$ of peak a < $T_R$ of peak b; $T_R$ in min)

## Tangent method (USP, ASTM):

$$R = \frac{2(T_{R(b)} - T_{R(a)})}{W_{B(b)} + W_{B(a)}}$$

## 5 Sigma method:

$$R = \frac{2.5(T_{R(b)} - T_{R(a)})}{W_{4.4(b)} + W_{4.4(a)}}$$

## Half-width method (Resolution used in Performance Report):

$$R = \frac{(2.35/2)(T_{R(b)} - T_{R(a)})}{W_{50(b)} + W_{50(a)}}$$

## Statistical method:

$$R = \frac{M1_{(b)} - M1_{(a)}}{W_{S(b)} + W_{S(a)}}$$

where:

$M1_{(x)}$ = mean retention time for peak x (1st Statistical Moment) [min]

$W_{B(x)}$ = base width for peak x [min]

$W_{4.4(x)}$ = width at 4.4% height for peak x [min]

$W_{50(x)}$ = width at 50% height for peak x [min]

$W_S(x)$ = width derived from statistical moments $= \sqrt{(M2)}$ for peak x [min] (see also "Statistical Moments" on page 244)

# Definitions for Reproducibility

For the statistical review of analytical data in terms of reproducibility the sequence is considered as a small random sample taken out of an infinite number of possible experimental results. To accomplish a complete set of results, an unlimited amount of sample material as well as time would be required. Strictly statistical data does only apply to a complete self-contained set or population of data. Therefore a prerequisite for such a treatment is that the selected sample can be assumed as representative for all data.

## Sample Mean M

The mean value M of a random sample consisting of N measurements is calculated from this limited set of N single observed values $X_i$ indexed with a consecutive counter i according to the formula:

$$M = \frac{\sum_{i=1}^{N} X_i}{N}$$

where:

N = number of discrete observations

$X_i$ = value of discrete observations indexed by i

## Sample Standard Deviation S

Consider a random sample of size N. The sample standard deviation S for the selected finite sample taken out of the large population of data is determined by

$$S = \sqrt{\frac{\sum\limits_{i=1}^{N}(X_i - M)^2}{N-1}}$$

The sample standard deviation S differs in two points from the standard deviation s for the whole population:

- instead of the real mean value only the sample mean value M is used and
- division by N-1 instead of N.

## Relative Standard Deviation RSD[%] (USP)

The relative standard deviation is defined as

$$RSD = 100\frac{S}{M}$$

## Standard Deviation of the Mean $S_M$

Let M be the sample mean and S the sample [or (N-1)] standard deviation. The standard deviation $S_M$ of the sample mean M is determined by

$$S_M = \frac{S}{\sqrt{N}}$$

This can be further illustrated by an example:

While the retention time of a certain compound may deviate slightly from the calculated mean value during one sequence, the data from another sequence may differ much more due to e.g. ambient temperature changes, degradation of the column material over time etc. To determine this deviation the standard deviation of the sample mean $S_M$ can be calculated according to the above formula.

# Confidence Interval CI

The confidence interval is calculated to give information on how good the estimation of a mean value is, when applying it to the whole population and not only to a sample.

The $100 \times (1 - \alpha)$ % confidence interval for the overall mean is given by

$$CI = t_{(\alpha/2);N-1} \cdot S_M$$

where:

$$t_{(\alpha/2);N-1}$$

percentage point of the t distribution table at a risk probability of $\alpha$)

For the extended statistics in the sequence summary report the 95% confidence interval may be used ($\alpha = 0.05$).

The t distribution (or 'student distribution') must be used for small sample volumes. In case of large sample volumes the results for the t distribution and the normal (gaussian) distribution do not differ any more. Therefore in case of 30 or more samples the normal distribution can be used instead (it would be very difficult to calculate the t-distribution for large numbers, the normal distribution is the best approximation of it).

95% Confidence Interval for 6 samples:

$1 - \alpha = 0.95$

$N = 6$

The correct value for t has to be taken from the t distribution table for 5 (N-1) degrees of freedom and for the value $\alpha/2$, being 0.025. This would give the following calculation formula for CI:

$$CI = 2.571 \cdot \frac{1}{\sqrt{6}} \cdot S_M$$

# Regression Analysis

Let

N = number of discrete observations

$X_i$ = independent variable, $i^{th}$ observation

$Y_i$ = dependent variable, $i^{th}$ observation

Linear function:

$$y_{(X)} = a + bX$$

Coefficients:

$$a = \frac{1}{\Delta_X}\left(\sum_{i=1}^{N} X_i^2 \cdot \sum_{i=1}^{N} Y_i - \left(\sum_{i=1}^{N} X_i \cdot \sum_{i=1}^{N} X_i Y_i\right)\right)$$

$$b = \frac{1}{\Delta_X}\left(N \cdot \sum_{i=1}^{N} X_i Y_i - \left(\sum_{i=1}^{N} X_i \cdot \sum_{i=1}^{N} Y_i\right)\right)$$

where:

$$\Delta_X = N \cdot \sum_{i=1}^{N} X_i^2 - \left(\sum_{i=1}^{N} X_i\right)^2$$

# Regression Coefficient

$$r = \frac{\left(N \cdot \sum_{i=1}^{N} X_i Y_i - \sum_{i=1}^{N} X_i \cdot \sum_{i=1}^{N} Y_i\right)}{\sqrt{\Delta_x \cdot \Delta_y}}$$

where:

$$\Delta_Y = N \cdot \sum_{i=1}^{N} Y_i^2 - \left( \sum_{i=1}^{N} Y_i \right)^2$$

## Standard Deviation (S)

$$S = \sqrt{\frac{\sum_{i=1}^{N} \{Y_i - a - bX_i\}^2}{N-2}}$$

# Internally Stored Double Precision Number Access

For validation purposes, it might become necessary to manually recalculate the ChemStation results such as calibration curves, correlation coefficients, theoretical plates, etc. When doing so the number format used in the ChemStation has to be taken into account.

For all numbers stored internally within the ChemStation, the "C" data type DOUBLE is used. This means that 14 significant digits are stored for each number. The implementation of this data type adheres to the Microsoft implementation of the IEEE standard for "C" data type and the associated rounding rules (see Microsoft documents Q42980, Q145889 and Q125056).

Due to the non-limited number of parameters that might be used for the calculation of the calibration table, it is not possible to calculate the exact error possibly introduced by the propagation and accumulation of rounding errors. Thorough testing with different calibration curve constructions however has shown that the accuracy of up to 10 digits can be guaranteed. Whereas the area, height and retention time repeatability of a chromatographic analysis usually has 3 significant digits, 10 significant digits within the calculations is sufficient. For this reason, the calibration, and other tables, display a maximum of 10 significant digits.

If an external (manual) calculation for validation is required, it is recommended that all digits used for the internal calculations are utilized. Using the displayed and/or rounded data for the external calculations might give results differing from the ChemStation due to rounding errors.

The following paragraph describes how to access all internally stored digits for numbers typically required for manual calculations. In all cases, a data file must be loaded and reported with the appropriate report style prior to execution of the listed command. All commands are entered on the ChemStation command line which may be enabled from the view menu. The information in file "C:\CHEM32\TEMP.TXT" may be viewed using NOTEPAD or a suitable TEXT editor.

### Raw Peak Information:

• Retention Time

- Area
- Height
- Width (integrator)
- Symmetry
- Peak Start Time
- Peak End Time

Use Command Line Entry:

```
DUMPTABLE CHROMREG, INTRESULTS,"C:\CHEM32\1\TEMP\
INTRES.TXT"
```

## Processed Peak Information:

- Measured Retention Time
- Expected Retention Time
- Area
- Height
- Width (integrator)
- Symmetry
- Half Width - Half Peak Height (Performance & Extended Performance)
- Tailing Factor (Performance & Extended Performance)
- Selectivity (Performance & Extended Performance)
- K` (Extended Performance)
- Tangent Peak Width (Extended Performance)
- Skew (Extended Performance)
- Theoretical Plates - Half Width (Performance & Extended Performance)
- Theoretical Plates - Tangent (Extended Performance)
- Theoretical Plates – 5-Sigma (Extended Performance)
- Theoretical Plates - Statistical (Extended Performance)
- Resolution - Half Width (Performance & Extended Performance)
- Resolution - Tangent (Extended Performance)

- Resolution – 5-Sigma (Extended Performance)
- Resolution - Statistical (Extended Performance)

Use Command Line Entry:

```
DUMPTABLE CHROMRES, PEAK,"C:\CHEM32\1\TEMP\PEAK.TXT"
```

## Processed Compound Information:

- Calculated Amount

Use Command Line Entry:

DUMPTABLE CHROMRES, COMPOUND,"C:\CHEM32\1\TEMP\
COMPOUND.TXT"

## Calibration Table Information:

- Level Number
- Amount
- Area
- Height

Use Command Line Entry:

```
DUMPTABLE _DAMETHOD, CALPOINT,"C:\CHEM32\1\TEMP\CALIB.TXT"
```

## Linear Regression Information:

- Y-Intercept (CurveParm1)
- Slope (CurveParm2)
- Correlation Coefficient

Use Command Line Entry:

```
DUMPTABLE _DAMETHOD, PEAK,"C:\CHEM32\1\TEMP\REGRESS.TXT"
```

# 12
# System Verification

This chapter describes the verification function and the GLP verification features of the ChemStation.

# Verification and Diagnosis Views

If supported by the configured instrument, for example, the Agilent 1100/1200 Series modules for LC, the ChemStation comprises two additional views to perform instrument verification and diagnosis tasks. For more information, see the online help system.

## System Verification

System verification is a key component in the routine use of an analytical instrument in a regulated laboratory. The GLP verification features of the ChemStation are designed to help you to prove that the software, or a relevant components of the software, are performing correctly, or were performing correctly at the time of a particular analysis.

The ChemStation verification function enables you to verify the correct operation of your ChemStation software. You can do this by reprocessing data files according to specific methods, and comparing the results with a pre-defined standard. The verification function is particularly important to prove the integrity of the integration and quantification results.

You can use the standard verification test, or define your own tests using your own method and data files to check the algorithmic software combinations used by your analysis methods. The verification test is a protected file and cannot be changed or deleted.

The Verification item in the Data Analysis view allows you to choose any of the following options:

• run a verification test in the database,

• define a new verification test and add it to the database, and

• delete a verification test from the database.

The How To section of the online help system describes how to perform these tasks. When running a ChemStation verification test, you can choose whether to run the entire test, or select a combination of parts.

Verification test results are saved in binary format to the default subdirectory: c:\CHEM32\1\Verify, together with the method and data files. The Verify subdirectory is at the same level as the sequence, methods and data subdirectories. You can send the results to a printer or to a file. The test results, including a combined verification test result, are rated as either pass or fail.

The following verification test components are available:

### Digital Electronics (Agilent 1100/1200 Series DAD only)

A test chromatogram is stored in the diode-array detector. This chromatogram is sent to the ChemStation after it has gone through the same preprocessing steps as normal raw data from the photodiodes. The resulting data are compared to original result data stored in the ChemStation for this test chromatogram. If there is a mismatch the test fails. This test ensures that the DAD electronics which do the data preprocessing are still functioning correctly. As a stored test chromatogram is used, the lamp or the diode array are not part of this test. They can be checked with the "DAD Test Function" on page 265.

### Peak Integration

The data file is integrated again using the original method. The results are compared to the original integration results stored in verification register. If they do not match, the test fails.

### Compound Quantification

The compounds in the data file are quantified again. The results are compared to the original quantification results stored in the verification register. If they do not match, the test fails.

### Report Printing

The original report is printed again.

The following page shows an example of a successfully completed verification test.

```
===================================================================
    ChemStation Verification Test Report
===================================================================
Tested Configuration:
    Component                         Revision
-------------------------------------------------------------------
  ChemStation for LC 3D ChemStation   B.01.01
  Microsoft Windows                   Microsoft Windows XP
  Processor                           Processor_Architecture_Intel
  CoProcessor                         yes

ChemStation Verification Test Details:
  Test Name : C:\CHEM32\1\VERIFY\DEFAULT.VAL
  Data File : C:\CHEM32\1\VERIFY\DEFAULT.VAL\VERIFY.D
  Method    : C:\CHEM32\1\VERIFY\DEFAULT.VAL\VERIFY.M
  Original Datafile            : VERIFY.D
  Original Acquisition Method  : VERIFY.M
  Original Operator            : Hewlett-Packard
  Original Injection Date      : 4/16/93 11:56:07 AM
  Original Sample Name         : Isocratic Std.

Signals Tested:
  Signal  1: DAD1 A, Sig=254,4 Ref=450,80 of VERIFY.D

ChemStation Verification Test Results:

  Test Module            Selected  For Test     Test Result
  -----------------------------------------------------------------
  Digital electronics test   No                     N/A
  Integration test           yes                    Pass
  Quantification test        yes                    Pass
  Print Analytical Report    No                     N/A

ChemStation Verification Test Overall Results:     Pass
```

# The GLPsave Register

The GLPsave register is saved at the end of each analysis when selected in the run time checklist. It contains the following information:

- signals,
- logbook,
- integration results table,
- quantification results table,
- instrument performance data, and
- data analysis method.

This register is a complete protected record, generated at the time of analysis. You can recall it at any time in the future as proof of your analytical methods.

The GLPsave Register option in the Data Analysis view enables you to review the GLPsave register file at any time. The file is protected by a checksum and is encoded into binary to ensure it is not changed.

In the dialog box used to select the GLPsave register for review, you can choose your review options from the following:

- load original method,
- load original signals,
- load instrument performance data,
- print original method,
- print original integration results,
- print original quantification results, and
- generate original report from the original method and signals.

You can use the GLP review function to show that chromatographic data are original, prove the quality of the analysis from the instrument performance data, and demonstrate the authenticity of the data interpretation.

For example, you can:

- reload and reprint the data analysis part of the method used at the time of the sample analysis to prove that the data evaluation, presented as the results of the analysis has not been modified in any way, and

- review without recalculating, the integration and quantification results to prove the authenticity of the report.

# DAD Test Function

Detector tests can be used as a step in the routine system validation of an analytical instrument in a regulated laboratory.

The DAD test assesses the performance of your diode array detector. When you select the DAD test from the Instrument menu (for LC3D and CE only) it checks the instrument for intensity and wavelength calibration. When you press Save the test results are automatically saved to the DADTest database, a register file called DADTest.Reg located in the default instrument directory.

## Review DAD Test Function

The **Review DAD Test** function in the data analysis **View** menu enables you to review the DADTest.Reg file at any time. The file is protected by a checksum, and is encoded into binary to ensure that it is not changed.

You can select any of the following parts of the DAD test for review:

**Show Holmium Spectra**   Plots all Holmium spectra listed in the DAD Test review table. The active spectrum is tagged.

**Show Intensity Spectra**   Plots all intensity spectra listed in the DAD Test review table. The active spectrum is tagged.

**Save as New Database**   If you change the lamp in your DAD you can reset the DADTest by deleting any unwanted test results from the table and then using the **Save as New Database** function.

**Show Selected Spectra**   Displays only spectra you selected in the table.

**Show Intensity Graph**   You can plot an intensity graph to give an indication of the life of the lamp in your diode array detector. The graph provides a function of maximum lamp intensity against time.

# Index

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# In This Book

This handbook describes various concepts of
the Agilent ChemStation. It is intended to
increase your understanding of how the
ChemStation works.

For information on using the ChemStation
please refer to the general help system and the
Online help "Tutorial".

**Agilent Technologies**